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13. ABSTRACT (Maximum 200 Words)
This research project examines the ability of synthetic oligonucleotides (ODN) containing immunostimulatory "CpG motifs' to trigger the innate immune system, thereby improving the host's ability to survive infection by biowarfare agents. Additional studies examining the ability of these CpG ODN to act as adjuvants when co-administered with vaccines being developed to prevent infection by biowarfare pathogens are also being pursued. Our initial results showed that CpG ODN protected mice against a variety of bacterial and viral pathogens, including Anthrax, Ebola, Listeria, and Tularemia. When used as vaccine adjuvants, these CpG ODN significantly boost antigen-specific IgG and type 1 cytokine production in both muring and non-human primate models.

Recent studies focused on 1) identifying the optimal type and number of BpG motifs needed to stimulate human immune cells, 2) establishing that these CpG ODN could protect against pathogen challenge in non-human primates and 3)that these CpG ODN could promote the induction of antifen-specific immune responses in non-human primates. Results indicate that CpG ODN need to contain multiple different CpG motifs to stimulate PBMC form diverse human donors. These ODN were found to protect rhesus macaques against pathogen challenge, and to augment the immunogenicity of co-administered vaccines (including AVA, rPA, and HKLV) in macaques. Serum transfer studies indicate that CpG ODN increase the magnitude and rapidity of the protective immune response elicited by vaccines against anthrax.

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4) INTRODUCTION

When first confronted with a pathogenic microorganism, the immune system rapidly mounts an "innate" immune response, characterized by the production of immunostimulatory cytokines and polyreactive antibodies (1;2). An inadequate innate response allows the infectious agent to overwhelm the host before a pathogen-specific immune response can develop. The research being funded by the U.S. Army Medical Research and Materiel Command examines the ability of synthetic oligonucleotides (ODN) containing immunostimulatory "CpG" motifs to trigger the innate immune system, thus improving the host's ability to survive infection and to generate a stronger and more rapid immune response to co-administered vaccines.

Early studies in our lab established that the innate immune system is activated by DNA of bacterial origin (3-6). Unmethylated CpG motifs present in bacterial DNA (but rare in mammalian DNA) mediate this stimulation. Synthetic oligonucleotides (ODN) containing unmethylated CpG motifs duplicate this effect (5-9). Within minutes of administration, CpG ODNs enter lymphoid cells and trigger cytokine, chemokine and Ig production.

Studies in mice showed that CpG ODN protected normal animals from infectious challenge by 10^2 - 10^5 LD_{50} of Listeria, Francisella, Malaria, and Leishmania for up to ten days (10-12). Subsequent reports established that CpG ODN could be co-administered with protein antigens to boost the resultant antigen-specific immune response (13-16). Ongoing research is focused on generating CpG ODN optimized for administration to humans to achieve these biological effects, and on demonstrating immunogenicity and protective efficacy in relevant non-human primate models.

5) BODY

A. Identifying the parameters that influence CpG ODN activity in humans.

1. Introduction

This lab previously documented that the murine immune system responds to synthetic CpG oligonucleotides (ODN) in which the CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines (5). Subsequent studies established that Toll-like receptor 9 (expressed by various types of immune cells) mediated this recognition of CpG motifs (17;18).

When CpG ODN that stimulate mice were tested on human PBMC, they induced little stimulation (18). This led us to hypothesize that TLR 9 expressed by rodents may have diverged from that expressed by primates over evolutionary time periods (18). Consistent with this hypothesis, we demonstrated that cell lines transfected with murine TLR9 responded to murine (but not human) CpG ODN, while cell lines transfected with human TLR9 responded to human (but not murine) CpG ODN (18).

To identify CpG motifs with optimal stimulatory activity in humans, we synthesized and tested hundreds of ODN that varied in size, CpG flanking region, and backbone composition. This work initially led to the identification of two distinct classes of CpG ODN (19). "K" type ODN were found to bind to and activate monocytes, macrophages and B cells, inducing them to proliferate and to produce IL-6 and/or IgM (19). In contrast, type "D" ODN primarily stimulated plasmacytoid dendritic cells (pDC),

improving their ability to present antigen and thus initiate an immune response (19). These two discrete types of ODN differed in their CpG flanking regions and in certain other structural elements.

Over the past several years, we focused our efforts on identifying ODNs capable of stimulating physiologically relevant immune responses in humans. Towards that end, many additional phosphorothioate ODNs were synthesized containing 1 - 5 different CpG motifs, and their activity was tested *in vitro* using PBMC from a dozen different normal healthy donors (20).

2. Effect of the number of CpG motifs on ODN activity.

Initial studies evaluated the effect of increasing the number of CpG motifs expressed by a single ODN. As seen in Table I, the average response elicited by ODNs that contained only one CpG motif was 25 ± 4 % of the maximum response elicited by the most stimulatory ODN. When two identical CpG motifs were present on a single ODN, the average level of immune activation rose to 47 ± 9% (p. <.01). Increasing the number of motifs to 3 or 4 resulted in 66 - 70% maximal stimulation (p. <.01, Table I). ODNs in which CpG motifs were incorporated at 5 sites were somewhat less active (49 ± 7%), suggesting that 3 - 4 was the optimal number of CpG motifs that could be effectively recognized in ODNs up to 35 bases in length. These results were not significantly affected by the spacing between motifs. We found that separating individual CpG motifs by 1, 2 or 3 nucleotides, or changing the length of an ODN by up to 8 bases, did not alter the relative magnitude of the immune response induced by different ODNs (data not shown).

Table I Effect of increasing the total number of CpG motifs expressed on the stimulatory activity of an ODN.

Total Number of	% maximal response					
CpG Motifs	Prolif	IL-6	IgM	IP-10	Avg	
1	30 ± 3	27 ± 2	25 ± 2	21 ± 1	25 ± 4	
2	54 ± 16	38 ± 5	59 ± 14	39 ± 7	47 ± 9	*
3	73 ± 13	57 ± 7	84 ± 12	50 ± 11	66 ± 13	*+
4	62 ± 7	68 ± 2	88 ± 5	63 ± 1	70 ± 11	*+
5	53 ± 4	56 ± 5	46 ± 2	41 ± 4	49 ± 7	*

The level of immune activation induced by 1 uM of 32 different ODNs (see Table I, series 1) was monitored in PBMC from ≥6 donors. IL-6, IgM and IP-10 levels in culture supernatants were measured by ELISA, while proliferation was measured by ^{3}H-thymidine incorporation. The maximum background vs immune stimulation observed in the samples studied was: IL-6; 0.2 vs 6 ng/ml, IgM; 0.3 vs 15 ug/ml, IP-10; 0.1 vs 2.2 ng/ml and proliferation 1,100 vs 27,100 cpm. To facilitate comparison between donors, the maximum response in each assay was set to 100, and the relative strength of each ODN then calculated by the formula: (response to ODN - background)/(maximum response - background) x 100%. The average and std deviation for each group is shown.

* Significantly greater than one motif, p <.01.
+ Significantly greater than two motifs, p < .01.

3. Immunostimulatory activity is influenced by the number of **different** motifs expressed by an ODN.

The effect of incorporating different CpG motifs within an individual ODN was then examined. As seen in Table II, ODNs containing multiple different motifs were significantly more immunostimulatory than those expressing a single motif multiple times. Molecules of the same size containing 3 - 4 different motifs were nearly three times as active as ODNs in which the same motif was present at 3-5 sites (p. <.01, Table II).

Table II Effect of increased CpG motif heterogeneity on an ODN's stimulatory activity

Number of Different CpG Motifs	% maximal response Prolif	IL-6	IgM	IP-10	Avg	
1	27 ± 2	26 ± 3	19 ± 4	21 ± 3	23 ± 4	
2	40 ± 16	44 ± 5	50 ± 14	42 ± 7	44 ± 4	*
3	63 ± 4	51 ± 5 **	78 ± 2	56 ± 4	62 ± 10	
4	69 ± 7	55 ± 2 **	74 ± 5	63 ± 1	65 ± 7	
5	48 ± 6	50 ± 3	42 ± 4	48 ±	54 ± 3	*

22 ODNs were synthesized that contained 1 - 5 different CpG motifs. The level of immune activation induced by 1 uM of each ODN was measured using PBMC from 6 - 10 donors, as described in the legend to Table I. The average level of immune stimulation induced by each group of ODNs is shown.
* Signficantly greater than one motif, p <.01.
+ Significantly greater than two different motifs, p < .01.

4. Immunostimulatory activity is influenced by the location of CpG motifs within an ODN.

The above findings are consistent with evidence showing that human PBMC can recognize and respond to an array of CpG motifs (19;21;22). The next experiment evaluated whether the 5' --> 3' order of these motifs affected the stimulation induced by an ODN. ODNs of similar length were synthesized in which either a relatively strong CpG motif (such as GGCGTT) or a relatively weak motif (such as CTCGAC) was inserted at any of 5 sites along the length of the ODN (site 1 being nearest the 5' end and site 5 being nearest the 3' end). ODNs containing the stronger motif at site 1 induced significantly greater immune activation than those containing a less active motif at the same position (63% vs 19%, p. <.01, Table III). This positional effect was observed in all assays of immune activation (proliferation, IgM, IL-6 and IP-10 production), indicating that ODNs with a strong CpG motif at the 5' end were broadly immunostimulatory. A similar but smaller positional effect was observed at site 3, where

inclusion of the more stimulatory motif generated significantly greater immune responses (59% vs 33%, p. <.02, Table III). At site 5 (nearest the 3' end), incorporation of a weaker motif generated ODNs of significantly greater activity (56% vs 42%, p. <.02). In contrast, the use of strong vs weakly stimulatory motifs at sites 2 and 4 had no significant effect on the activity of an ODN.

Table III Effect of CpG motif position on an ODN's stimulatory activity

Site	Motif	Prolif	IL-6	IgM	IP-10	Avg
1	Strong	70 ± 9	67 ± 5	63 ± 7	64 ± 7	63 ± 7 *
1	Weak	21 ± 1	16 ± 3	18 ± 2	21 ± 1	19 ± 2
1	Control	26 ± 5	23 ± 5	21 ± 3	13 ± 1	21 ± 6
2	Strong	49 ± 8	45 ± 6	42 ± 6	44 ± 10	45 ± 2
2	Weak	42 ± 7	65 ± 9	36 ± 4	40 ± 5	46 ± 11
3	Strong	61 ± 10	66 ± 7	50 ± 7	60 ± 10	59 ± 6 *
3	Weak	35 ± 3	38 ± 3	38 ± 3	22 ± 2	33 ± 7
4	Strong	42 ± 4	60 ± 7	47 ± 3	47 ± 8	49 ± 8
4	Weak	51 ± 7	50 ± 5	61 ± 6	37 ± 3	50 ± 8
5	Strong	45 ± 7	49 ± 6	40 ± 3	47 ± 7	42 ± 7 *
5	Weak	53 ± 5	52 ± 4	60 ± 6	45 ± 4	56 ± 9

ODNs containing a strong, weak or control (non-CpG) motif at sites 1 - 5 were synthesized. The level of immune activation induced by 1 uM of each ODN was measured in PBMC from 6 donors, as described in the legend to Table I. The average level of immune stimulation induced by all ODNs with a specific motif at each site is shown.

* Significant difference between strong vs weak motifs, p <.02.

B. Establishing a non-human primate model for studying the *in vivo* activity of CpG ODN.

1. Introduction

Previous studies established that human PBMC respond to two structurally distinct classes of CpG ODN (19). "D" type ODN triggered the secretion of IFNα and IFNγ (19) whereas "K" ODN induced human PBMC to proliferate and secrete IL-6 and IgM (Fig1, (19), and data not shown). Analysis of several hundred CpG ODN identified several "D" and "K" ODN that strongly activated human PBMC (19). Some of these ODN (Table IV) were tested for their ability to stimulate PBMC from rhesus macaques.

Table IV: Sequence and backbone of "D", "K" and control ODN.

D19: GGTGCAT**CG**ATGCAGGGGGG
D29: GGTGCAC**CG**GTGCAGGGGGG
D35: GGTGCAT**CG**ATGCAGGGGGG
D122: GGTGCATTGATGCAGGGGGG
K3: AT**CG**ACTCT**CG**AG**CG**TTCTC
K123: T**CG**TT**CG**TTCTC
K23: T**CG**AG**CG**TTCTC
K163: T**TG**AG**TG**TTCTC
AA3M: GGGCATGCATGGGGGG

Bases on a gray background are phosphodiester while those on a white background are phosphorothioate.

2. Response of macaque PBMC to human CpG ODN.

Since CpG ODN that are most active in humans are poorly immunostimulatory in mice, a relevant animal model was need to study the activity of "human" ODNs *in vivo*. Towards this end, the response of rhesus PBMC to "D" and "K" ODN was evaluated. Results show that macaque PBMC were activated to produce TNFa by the same "D" ODN that stimulate human PBMC ($p<0.002$, Fig 1). In contrast, neither "K" ODN, nor control ODN that are structurally similar to "D" but lack the critical CpG dinucleotide, had this effect.

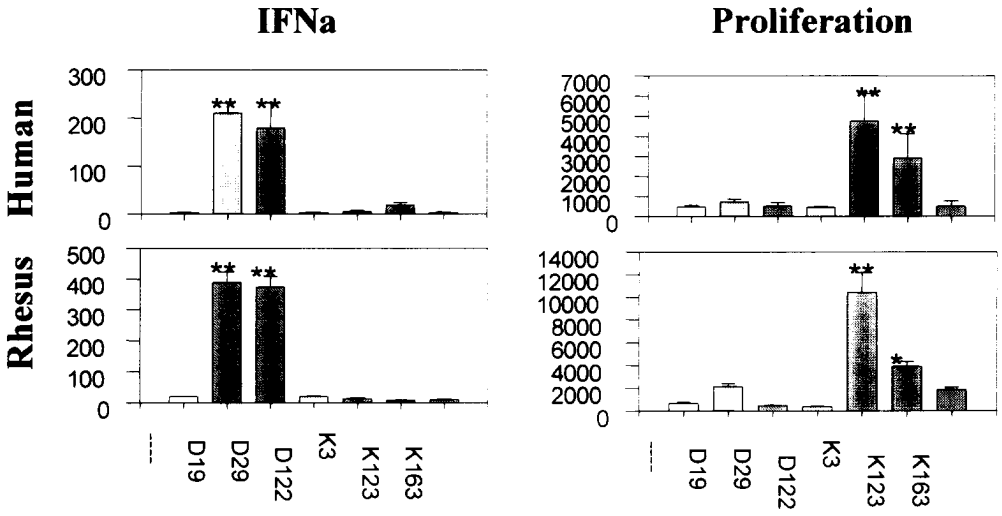

Figure 1. Response of primate PBMC to "K" and "D" ODN.

PBMC from 8-20 normal human donors and 20 rhesus macaques were stimulated for 72 hr with 3 μM of "K", "D" or control ODN (in which the critical CpG

motifs were inverted or replaced with TpG). IFNa levels in culture supernatants (units/ml) were determined by ELISA, while cell proliferation (DPM) was assessed by $[H]^3$ thymidine uptake. Note that "D" ODN induce the secretion of IFNα while "K" ODN induce cell proliferation (and IL-6 production, data not shown). All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. * $p < 0.05$; ** $p < 0.01$.

Proliferation and IL-6 secretion were used to compare the response of macaque and human PBMC to "K" ODN (Fig 1). PBMC from both species were stimulated by "K" ODN to proliferate ($p < 0.002$) and secrete IL-6 ($p < 0.01$), whereas controls of the same structure as "K" ODN but lacking the critical CpG motif failed to trigger immune stimulation. These findings demonstrate that the pattern of reactivity of PBMC from rhesus macaques (N = 20) and humans (N = 8-20) to "K" and "D" ODN is quite similar.

Ongoing studies in our lab indicate that individual humans and monkeys vary in their response to specific "K" and "D" sequences. Indeed, no single "D" or "K" motif is optimally stimulatory in all donors (23). However, mixtures of ODN were identified that strongly stimulated PBMC from all human donors. These mixtures were tested on PBMC from macaques and found to be uniformly active (Fig 2). Subsequent *in vivo* studies were conduced with these ODN mixtures.

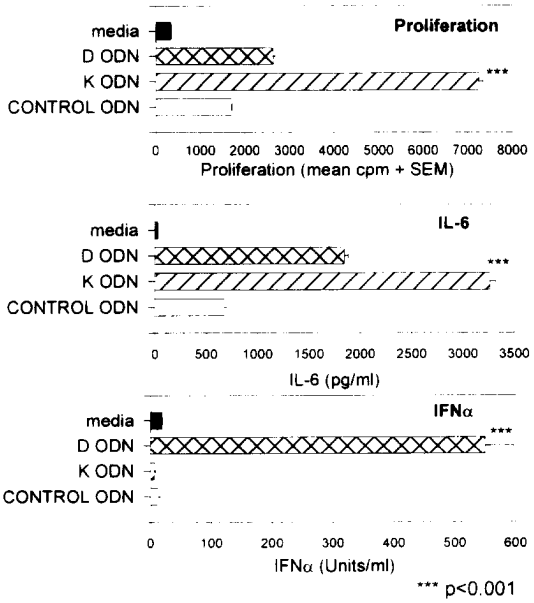

Figure 2. Macaque PBMC respond to CpG ODN mixtures optimized for human use.

PBMC from rhesus macaques (N = 12-20) were stimulated *in vitro* for 72 hr with a mixture of D19, D29 and D35 (1 μM each) or K3 and K123 (1.5 μM each). D122 and K163 were used in the control ODN mixture. Levels of IL-6 and IFNα in culture supernatants were measured by ELISA, while proliferation was measured by $[H]^3$-

thymidine uptake. Statistical significance was determined by ANOVA of the normalized data. ** $p<0.01$.

C. Immunoprotective activity of CpG ODN in Rhesus Macaques

1. Introduction

The results described above support the *in vivo* testing of CpG ODN proposed for human use in rhesus macaques. Initial studies utilized a cutaneous Leishmaniasis infection model. This model was selected because I) previous studies established that CpG ODN prevent leishmania infection in mice and II) cutaneous leishmania is a self-limited disease in rhesus monkeys and thus approved for study at CBER (24).

2. Model system.

To determine whether CpG ODN could protect primates from leishmania infection, rhesus macaques were treated i.d. with 500 ug of either D or K CpG ODN. Treatment was adminstered 3 days before and 3 days after intradermal challenge with 10^8 metacyclic promastigotes of *L. amazonensis*. This challenge induced the development of cutaneous *Leishmania* lesions resembling those that develop naturally in infected humans (Fig 3). In untreated monkeys the peak surface area was of 14 $\pm$ 10 mm^2 on day 22. As shown in Fig. 3, treatment of the macaques with D ODN, but not with K ODN, significantly reduced the size of these cutaneous lesions ($p<0.001$). Thus, treatment of primates with CpG ODN type D results in protection from local challenge with pathogenic Leishmania parasites. This finding represents the first example of CpG ODN increasing the resistance of primates to an infectious pathogen.

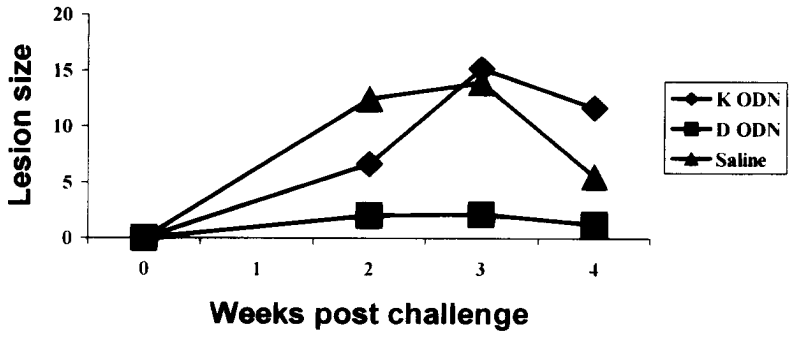

Fig 3 Effect of CpG ODN treatment on Leishmania lesions.

Cutaneous lesions in macaques treated 500 µg of a mixture of (D19, D29 + D35) (N=6) , (K3 + K123) (N=6) or saline i.d. 3 days before and 3 days after an infectious challenge with 10^7 *l. amazonensis* metacycle promastigotes (PH8). The average size of the resultant lesions is shown as the mean area (calculated as mean diameter/2)^2 x pi). Note that macaques treated with "D"ODN had significantly smaller lesions ($p<0.05$).

D. Use of CpG ODN as immune adjuvants in non-human primates.

1. Introduction

Owing to the difficulty of using lethal agents for challenge studies involving rhesus macaques, the experimental system described above was used to analyze the ability of CpG ODN to act as an immune adjuvant. Specifically, we analyzed whether the addition of CpG ODN to HKLV could improve the immunogenicity of that vaccine.

2. CpG ODN improve the immunogenicity of Heat-Killed Leshmania vaccine (HKLV).

Previous human clinical trials showed that HKLV in alum is a safe but poorly immunogenic vaccine (25). We immunized and boosted macaques with a mixture of HKLV in alum plus CpG ODN. PBMC from these animals were isolated 10 days post boost and re-stimulated *in vitro* with leishmania antigen for 72 hr. As seen in Fig 4, both "K" and "D" ODN significantly increased the number of PBMC triggered to secrete IFNγ ($p<0.05$). In contrast, animals immunized with alum-adsorbed HKLV alone showed no increased IFNg production when compared to unimmunized controls.

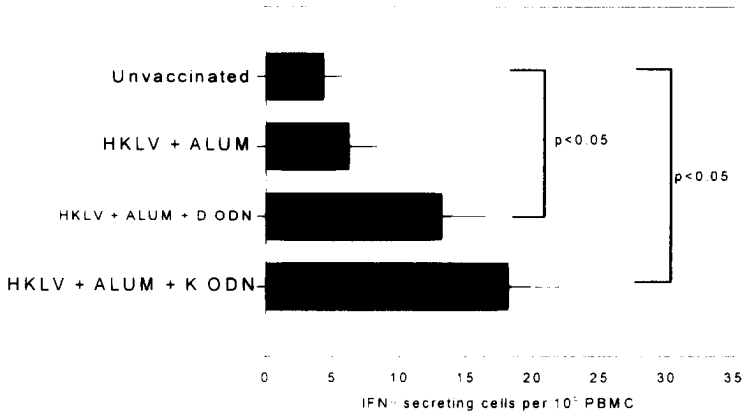

Figure 4 IFNγ production by PBMC from macaques immunized with alum-adjuvanted HKLV plus ODN.

Rhesus macaques were immunized and boosted with 250 µg of HKLV-alum alone (N=6), or combined with 500 µg of a mixture of "D"(D19, D29 and D35) (N=5) or "K" (K3 and K123) (N=5) ODN. PBMC from these animals were incubated with 25 µg of Leishmania antigen and analyzed *in vitro* for IFNγ production by ELISPOT assay. Animals immunized with HKLV plus "K" or "D" ODN had significantly more IFNγ secreting cells than unvaccinated controls as determined by a one way ANOVA ($p<0.05$).

The critical measure of an antigen/adjuvant combination is its ability to induce protective immunity. Vaccinated animals were therefore challenged with 10^7 *L. major*

metacyclic promastigotes. Animals vaccinated with HKLV-alum alone developed typical cutaneous lesions with a peak surface area of 300 $\pm$ 60 mm^2 26 days after challenge (Fig 5). Monkeys vaccinated with HKLV-alum plus "K" ODN developed lesions of similar size, although the peak lesion formation was slightly delayed. Animals immunized with HKLV-alum plus "D" ODN had significantly smaller lesions (maximal size 80 $\pm$ 13 mm^2, $p<0.05$), consistent with a reduced parasite burden (24).

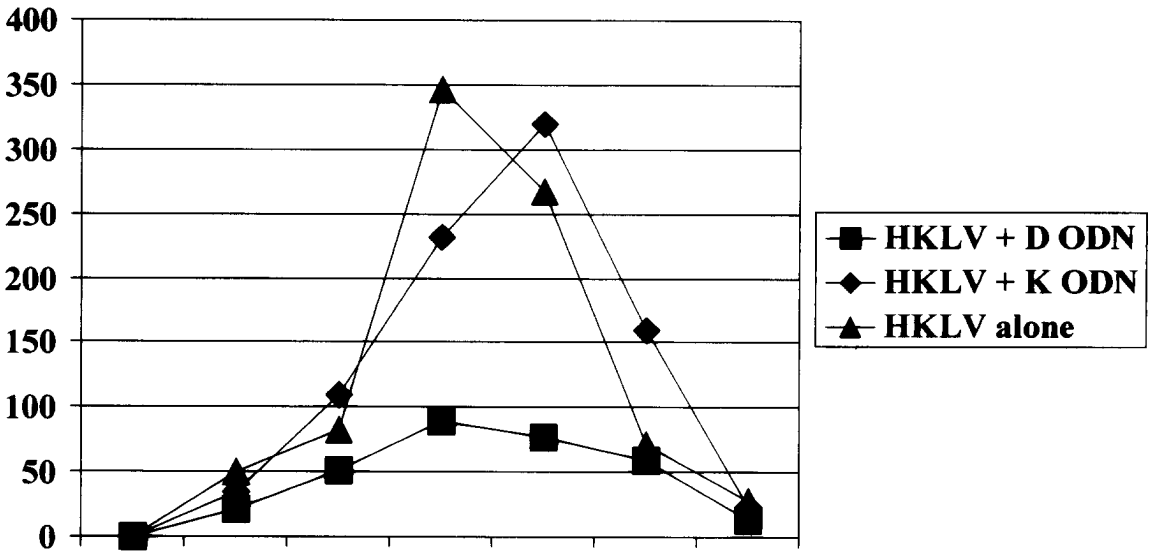

Fig 5 Cutaneous lesions in macaques vaccinated with alum-adjuvanted HKLV plus ODN.

Rhesus macaques were primed s.c. with 250 µg of alum-adjuvanted HKLV alone (N=6) or combined with 500 µg of a mixture of ODN (D19, D29 and D35, N=5) or (K3 and K123, N=5) and boosted 4 weeks later. On wk 14, the monkeys were challenged with 10^7 metacyclic promastigotes. The average size of the lesions on the forehead (the site of challenge) is shown as the mean area (calculated as mean diameter/2)^2 x pi). Note that macaques immunized with HKLV plus "D"ODN had significantly smaller lesions ($p<0.01$).

E. Use of CpG ODN to improve antrax vaccination

1. Introduction

Based on the results presented in section "D", we examined whether CpG ODN could improve the rapidity and titer of the protective immune response elicited by AVA,

the licensed anthrax vaccine. Towards that end, immunization in 3 animal models (mice, guinea pigs and rhesus macaques) was examined.

2. CpG ODN improve the immunogenicity of AVA in mice.

A/J mice were immunized with increasing doses of AVA ± 20 ug of CpG ODN (a safe and biologically active dose of ODN (26)). As the dose of vaccine was increased, animals co-immunized with CpG ODN rapidly generated significantly higher titers of IgG anti-PA Abs than mice immunized with AVA alone ($p < .01$, Fig 6). Mice vaccinated with ≥8 ug of AVA ± CpG ODN were challenged one week later with ≥100 LD_{50} of spores from the non-encapsulated Sterne strain of *B anthracis*. A significantly greater fraction of mice immunized with AVA + CpG ODN survived challenge (29/39, 74%) than those immunized with AVA alone (12/46, 26%, $p < .01$) or AVA + control ODN (2/12, 17%, $p < .01$).

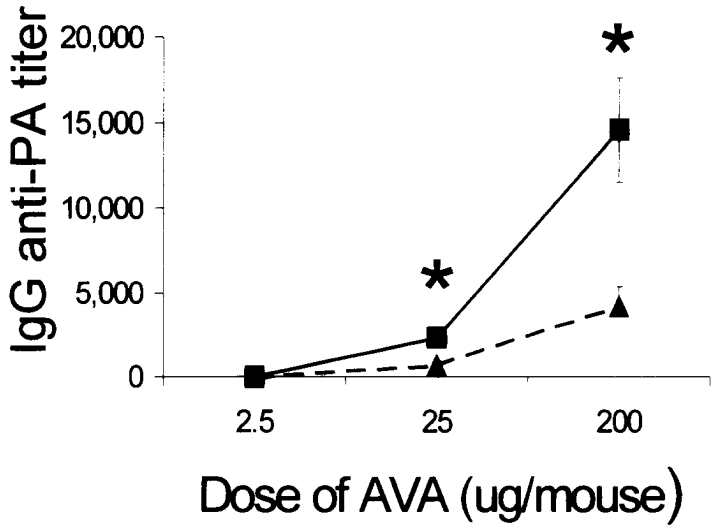

Fig 6 CpG boost the immunogenicity of AVA in mice

Ten A/J mice/group were immunized with AVA alone (▲) or combined with CpG ODN (■). Serum IgG anti-PA titers were measured on day 14.
* Significantly greater than AVA alone, $p < .05$.

3. CpG ODN improve the survival of AVA vaccinated guinea pigs.

To confirm and extend these findings, a guinea pig challenge model was employed (27). Normal guinea pigs succumb rapidly to challenge by 50 LD_{50} of encapsulated Ames strain anthrax spores (Table V). Immunization and boosting with AVA alone improves their survival rate, although most animals still die from infection (Fig 2). By

comparison, 75% of animals immunized and boosted with AVA + CpG ODN survive challenge (p = .05).

Table V Effect of CpG ODN plus AVA on the survival of guinea pigs challenged with anthrax

	# Surviving/total	% Surviving
Untreated	1/28	3.6
AVA	15/32	46.9
AVA + CpG ODN	23/31*	74.2

Guinea pigs were immunized on day 0 and boosted on week 4 with 0.5 ml AVA plus 100 - 300 ug of CpG ODN known to be active in this species. Six weeks later they were challenged IM with 50 LD_{50} *B. anthracis* Ames spores. Percent survival and the number of animals surviving/total is shown.

* Significantly improved survival compared to animals immunized with AVA alone, p = 0.05.

4. CpG ODN improve the immunogenicity of AVA in rhesus macaques.

Due to evolutionary divergence in CpG recognition between species, CpG motifs that are highly active in rodents are poorly immunostimulatory in primates, and vice versa (19;22;28). Since rhesus macaques and humans respond to the same CpG ODN, macaques provide an excellent model for examining the adjuvant activity of ODNs planned for human use (19;29).

In preliminary studies, the combination of CpG ODN with AVA significantly increased the maximum, average, and long-term IgG anti-PA response of macaques (data not shown). To confirm these findings, AVA was co-administered with GMP-grade CpG ODN 7909 (currently undergoing clinical trials for cancer therapy). As seen in Fig 7,administering the normal human dose of AVA with 250 ug of ODN 7909 stimulated on average a 6-fold higher serum IgG anti-PA response than AVA alone (p. <.001). Significantly increased anti-PA titers developed within 11 days of vaccination (p. <.05), consistent with CpG ODN inducing a more rapidly protective response than AVA alone.

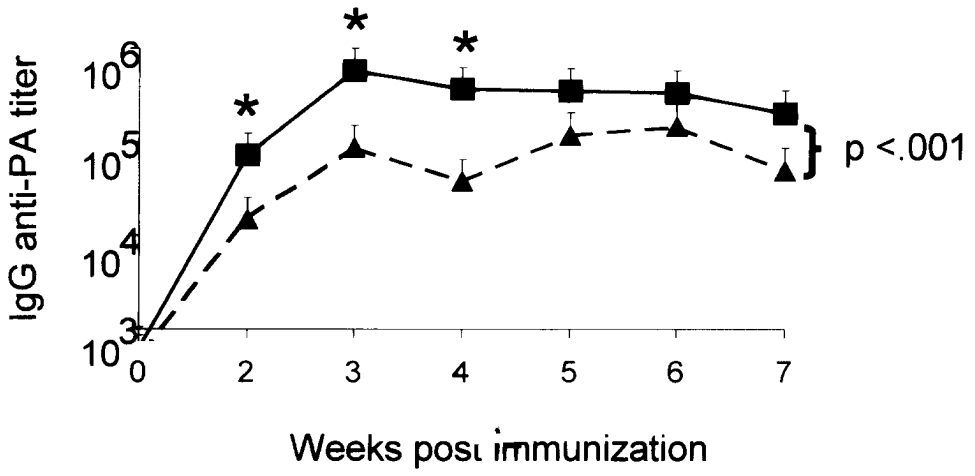

Fig 7 CpG ODN boost the immunogenicity of AVA in rhesus macaques.

Rhesus macaques (5/group) were immunized s.c. with 0.5 ml ± 250 ug of CpG ODN 7909. Results represent the geometric mean IgG anti-PA titer calculated by analyzing serum from each animal independently at each time point.

* Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $p < .05$.

5. Efficacy of the macaque anti-PA response.

The critical measure of an antigen-adjuvant combination is its ability to induce protective immunity. In preliminary studies, macaques immunized with AVA plus CpG ODN mounted a stronger and longer-lasting immune response when "challenged" with the attenuated Sterne strain of *B. anthracis* than did animals immunized with AVA alone (data not shown). Due to restrictions on the use of macaques in lethal anthrax challenge trials, other methods were used to examine whether protective immunity was elicited. Measuring the anthrax toxin neutralization capacity of serum provided one surrogate for protection (30). On day 11 post immunization, serum from macaques immunized with AVA + 7909 had 17-fold greater toxin neutralizing activity than serum from animals immunized with AVA alone (434 ± 209 vs 25 ± 5, $p < .03$). More dramatic evidence of protection was obtained by transferring serum from immunized macaques to A/J mice. Ninety percent of the recipients of pre-immune serum or serum from AVA immunized macaques succumbed to challenge by *B. Anthracis* Sterne strain spores (Fig 8). By comparison, serum from macaques 11 days post immunization with AVA plus CpG ODN protected nearly half of recipient mice from ≥30 LD_{50} of Sterne strain spores ($p. < .03$).

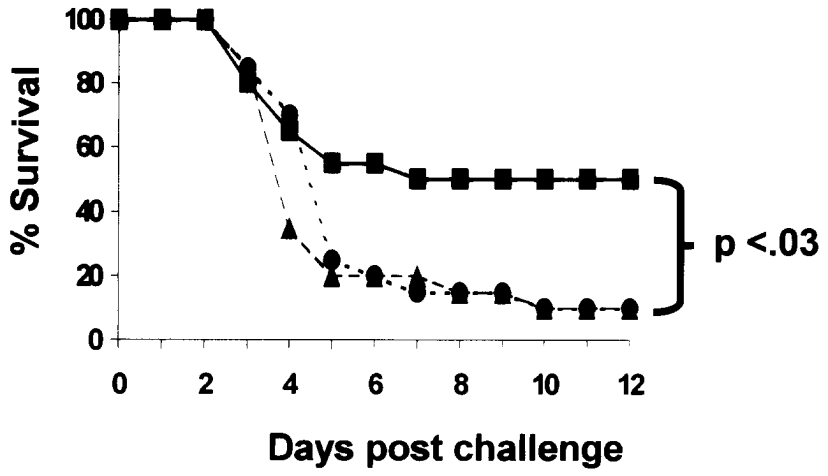

Fig 8 Serum from macaques immunized with CpG ODN plus AVA protect naive mice from anthrax spore challenge.

Pre-immune serum (○), and serum collected 11 days after vaccination of rhesus macaques with AVA alone (▲) or combined with CpG ODN (■) was pooled and injected i.p. into A/J mice (0.1 ml serum/recipient). The following day, mice were challenged with 50 - 60 LD_{50} of Sterne strain anthrax. The combined results of two independent experiments involving 10 recipients/treatment/experiment are shown.

6) KEY RESEARCH ACCOMPLISHMENTS

1) We determined the number, type and location of CpG motifs within an ODN that optimally stimulate the production of Ig and cytokines by human PBMC.

2) We demonstrated that K and D type CpG ODN differentially activate PBMC from humans, and that this pattern of reactivity is mirrored in the response of rhesus macaques.

3) We showed that same classes of CpG ODN that stimulated human PBMC *in vitro* were active in rhesus monkeys *in vivo*. These ODN were shown to act both as immune adjuvants and to provide protection against infection by Leishmania.

4) We established for the first time that CpG ODN designed for human use could protect non-human primates from infectious challenge (using a leishmania model and rhesus macaques).

5) We documented that CpG ODN could act as adjuvants when combined with both HKLV and AVA vaccines. The combination of CpG ODN plus vaccine resulted in higher antigen-specific antibody titers, and improved protection, in mice, guinea pigs and rhesus macaques.

7) REPORTABLE OUTCOMES

A) Manuscripts

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B) Oral Presentations

1) Speaker, "Immunostimulatory and immunosuppressive activity of DNA",LFKRI, New York, NY, 2001.

2) Speaker, "Immunoprotective activity of CpG Oligonucleotides", 2nd Annual Symposium on the Immunology of Infectious Disease, USUHS, Bethesda, MD, 2001.

3) Speaker, "CpG ODN induce protection against biothreat pathogens", 2nd International Symposium on CpG Oligos, Ameilia Island, FL, 2001.

4) Speaker, "CpG adjuvant activity", Vaccines of the future: from rational design to clinical development. Paris, France, 2001.

5) Speaker, "Role of CpG stimulatory and inhibitory motifs in innate and cognate immune responses", Keystone Symposium, Breckenridge, CO. 2002.

6) Speaker, "CpG Oligonucleotides as Immune Adjuvants", IMV 2002 Vaccine Conference, Prague, Czechoslovakia

7) Co-chairman and speaker, "Innate immunity Session", Trudeau Institute Symposium on Immunity to Viral Infections, Saranac Lake, NY, 2002.

8) Speaker, "Induction of protective immune responses by CpG oligodeoxy-nucleotides", Biodefense: Research, Technologies and Applications, McLean, VA 2002.

9) Speaker, "Suppressive Oligonucleotides regulate CpG Induced Immune Activation", 6th NIH Symposium on Therapeutic Oligonucleotides. Bethesda, MD 2002.

10) Chairman, Workshop on "Evaluating Nucleic Acid Based Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

11) Plenary Lecture, "Science Based Regulation of DNA Vaccines", 7th Symposium on Regulatory and Analytical Sciences, San Francisco, CA 2003.

12) Lecture, "Use of CpG Oligonucleotides for Biodefense", University of Texas Medical Branch, Galveston, Tx, 2003

C) Patents or patent applications

1) Use of Sterically Stabilized Cationic Liposomes to Efficiently Deliver CpG Oligonucleotides *in vivo* DHHS Reference No. E-215-01/0.

2) Novel Method for Rapidly Generating Mature Dendritic Cells from Peripheral Blood Monocytes and Myeloid Precursors DHHS Reference No. E-214-01/0

3) Method of Treating and Preventing Infections in Immunocompromised Subjects with Immunostimulatory CpG Oligonucleotides DHHS Reference No. E-153-02/0.

4) Multiple CpG Oligodeoxynucleotides and their Use to Induce and Immune Response. DHHS Reference No. E-078-00/1.

8) CONCLUSIONS

Studies conducted over the past two years as part of this project focused on identifying CpG ODN that strongly activate human PBMC *in vitro*, and showing that these ODN have activity *in vivo* in non-human primates. Results of this work clarified the rules governing CpG recognition by cells expressing Toll-like receptor 9, facilitating the development of optimally stimulatory CpG ODN. They also led to the synthesis of CpG ODN mixtures that strongly stimulate PBMC from virtually all human and rhesus donors tested.

Results of *in vivo* studies documented that optimized ODN mixtures activate the immune system of rhesus monkeys. As stand alone agents, we found that CpG ODN significantly increased host resistance to leishmania challenge, the first example of such protection in primates. Of equal importance, we established that CpG ODN (both the ODN mixtures described above and GMP-grade ODNs currently undergoing human clinical testing) could boost the immune response to a co-administered vaccine. Studies involving the HKLV vaccine showed that CpG ODN improved protection in a relevant primate model, while studies involving AVA showed that CpG ODN increased pathogen-specific Ab production and protection in mice, guinea pigs and rhesus macaques. Both the speed and the magnitude of these protective responses were significantly boosted by CpG ODN co-administration. These results suggests that combining CpG ODN with AVA could accelerate the development of protective immunity in individuals are risk of exposure to anthrax. No severe adverse events were observed in any of the CpG ODN treated primates. These findings form the basis for an IND recently submitted to the FDA requesting permission to initiate a phase I clinical trial of AVA plus CpG ODN to improve the induction of protective immunity against anthrax.

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10) APPENDICES

CpG Oligodeoxynucleotides Protect Normal and SIV-Infected Macaques from *Leishmania* Infection[1]

Daniela Verthelyi,[2,3]* Mayda Gursel,* Richard T. Kenney,† Jeffrey D. Lifson,‡ Shuying Liu,§ Joan Mican,§ and Dennis M. Klinman*

Oligodeoxynucleotides containing CpG motifs (CpG ODNs) mimic microbial DNA and activate effectors of the innate immune response, which limits the spread of pathogens and promotes an adaptive immune response. CpG ODNs have been shown to protect mice from infection with intracellular pathogens. Unfortunately, CpG motifs that optimally stimulate humans are only weakly active in mice, mandating the use of nonhuman primates to monitor the activity and safety of "human" CpG ODNs in vivo. This study demonstrates that CpG ODN treatment of rhesus macaques significantly reduces the severity of the lesions caused by a challenge with *Leishmania*. *Leishmania* superinfection is common in immunocompromised hosts, particularly those infected with HIV. This study shows that PBMCs from HIV-infected subjects respond to stimulation with CpG ODNs. To determine whether CpG ODNs can protect retrovirus-infected primates, SIV-infected macaques were treated with CpG ODNs and then challenged with *Leishmania*. Both lesion size and parasite load were significantly reduced in the CpG-treated animals. These findings support the clinical development of CpG ODNs as immunoprotective agents in normal and HIV-infected patients. ***The Journal of Immunology*, 2003, 170: 4717–4723.**

Stimulation of the innate immune system by determinants expressed by infectious microorganisms serves to limit the early spread of a pathogen while promoting the development of Ag-specific immunity (1). Unmethylated CpG motifs present at high frequency in bacterial but not vertebrate DNA are recognized by Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells (DCs)[4] (2–4). The interaction of Toll-like receptor 9 with CpG motifs triggers an immune cascade, resulting in improved Ag uptake/presentation by APCs and the secretion of polyreactive Ig, chemokines, and cytokines by B cells, NK cells, DCs, and monocytes (5, 6). Synthetic oligodeoxynucleotides (ODNs) expressing CpG motifs mimic the immunostimulatory activity of bacterial DNA (7).

There is considerable interest in developing novel agents that improve host resistance against infectious microorganisms. Studies in murine models indicate that CpG ODNs facilitate host clearance of infectious pathogens such as *Leishmania*, *Listeria*, and *Francisella tularensis* (8–11). Protection is observed even in T cell-depleted immunodeficient mice, raising the possibility that CpG ODNs might also help immunocompromised patients resist opportunistic infections (9). Exploration of this issue using murine models is of limited value, however, because the precise CpG motifs that are most active in rodents are poorly immunostimulatory in primates (due to evolutionary divergence in CpG recognition) (12–14).

Two types of CpG ODNs that activate PBMCs from human and nonhuman primates have been identified (14–16). "D" type ODNs trigger plasmacytoid DCs to secrete IFN-α (17), monocytes to mature into functionally active DCs (18), and NK cells to secrete IFN-γ (14, 17), whereas "K" type ODNs primarily stimulate B cells and monocytes to proliferate and secrete IgM, IL-10, and IL-6 (15, 17). To date, the ability of these ODNs to stimulate immune cells from immunocompromised donors or to provide protection in vivo in a relevant challenge model has not been examined.

HIV-infected patients have multiple defects in immune reactivity, reflecting a loss in the number and/or function of $CD4^+$ T cells, NK cells, macrophages, and DCs (19–22). These defects increase their susceptibility to opportunistic infections, which in turn accelerates the course of AIDS (23). One such opportunistic pathogen is *Leishmania* (24). Leishmaniasis is a protozoan infection that causes skin lesions ranging in size from small spontaneously healing papules to large mutilating ulcers (24). The course of infection is influenced by the nature of the host's immune response, with Th1-type immunity (high levels of IFN-γ) being associated with reduced parasite load and smaller lesions, whereas Th2-type immunity (increased IL-10) favors more severe disease. HIV-infected patients are more susceptible to infection and typically develop the more aggressive visceral form of the disease (25).

The current study was undertaken to determine whether PBMCs from immunocompromised, retrovirus-infected primates can respond to D and K CpG ODNs. The protective activity of CpG ODNs in *Leishmania*-infected rhesus macaques was then examined (26). Results indicate that CpG ODNs enhance host resistance to infectious challenge by *Leishmania*, even when the subject is immunosuppressed.

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[4] Abbreviations used in this paper: DC, dendritic cell; ODN, oligodeoxynucleotide; i.d., intradermally.

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Materials and Methods

Oligodeoxynucleotides

ODNs were synthesized by the Center for Biologics Evaluation and Research Core Facility. Sequences used were as follows (phosphorothioate bases in uppercase and phosphodiester bases in lowercase): D19, GGtg catcgatgcagGGGGG; D35, GGtgcatcgatgcaggggGG; D29, GGtgcaccggtg cagGGGGG; K3, ATCGACTCTCGAGCGTTCTC; K123, TCGTTTGT TCT; and K23, TCGAGCGTTCTC. Control ODNs included D122 (GGtgcattgatgcagGGGGG) and K163 (TGCAGGCTTCTC). All ODNs had <0.1 endotoxin U of endotoxin per milligram of ODN as assessed by a *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Walkersville, MD). Previous studies from our lab have shown that individual humans and monkeys vary in their responses to specific K and D sequences. Indeed, no single D or K motif is optimally stimulatory in all donors (27). However, mixtures of ODNs were identified that strongly stimulated PBMCs from all human donors (15, 27). These D or K ODN mixtures were used in our in vivo studies in macaques.

Human PBMCs

Buffy coats from healthy blood donors were obtained from the National Institutes of Health Department of Transfusion Medicine. PBMCs from HIV-infected subjects were obtained from the Infectious Diseases Section of the Department of Transfusion Medicine at the National Institutes of Health Blood Bank and from the National Institute of Allergy and Infectious Diseases (National Institutes of Health) after appropriate consent. Their clinical characteristics are summarized in Table I.

Rhesus macaques

Healthy 3-year-old rhesus macaques (*Macaca mulata*) were obtained from the Food and Drug Administration colony in South Carolina. All studies were Institutional Animal Care and Use Committee approved and were conducted in an American Association for the Accreditation of Laboratory Animal Care accredited facility. Animals were monitored daily by veterinarians. No systemic or local adverse reactions to CpG ODNs were observed. Treatments were administered and peripheral blood samples obtained from ketamine-anesthetized animals (10 mg/kg, Ketaject; Phoenix Pharmaceuticals, St. Joseph, MD).

Mononuclear cell preparation

Mononuclear cells were isolated by density gradient centrifugation of PBMCs over Ficoll-Hypaque as described (14). Cells were washed three times and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 5×10^5 cells/well in the presence of 1–3 μM ODN. Supernatants were collected after 72 h and tested by ELISA for cytokine and Ab levels.

Macaque treatment groups and protocol

Study no. 1. Eighteen healthy rhesus macaques (six per group) were challenged on the forehead on day 0 with 10^7 *Leishmania amazonensis* (PH8) metacyclic promastigotes intradermally (i.d.) as previously described (15, 28). Three days before and 3 days after challenge, 500 μg of a mixture of K or D ODNs was administered i.d. at the same site. Control monkeys ($n = 6$) received saline. Animals developed a typical self-limited lesion in situ, characterized by erythema, induration, and ulceration. Lesion size, which reflects the severity of infection, was measured weekly in a blinded fashion.

Study no. 2. Fourteen rhesus macaques chronically infected with SIVmac239 were obtained by transfer to the current study after completion of a separate research protocol fully described by Lifson et al. (29). Six healthy macaques were included in the study as controls. The macaques were superinfected with 10^7 *L. major* metacyclic promastigotes i.d. Three days before and 3 days after infection, they were treated i.d. with 250 μg of D ($n = 4$) or K ($n = 4$) ODNs at the site of challenge. Healthy monkeys treated with saline ($n = 6$) and SIV-infected monkeys that received control ODN ($n = 3$) or saline ($n = 3$) served as controls. Lesion size and SIV viremia were measured weekly. Three monkeys (one from control ODN and two from K-treated groups) were euthanized during the study due to weight loss and/or uncontrollable diarrhea. On day 56, the lesions were biopsied, the surviving animals were euthanized, and the local and systemic parasitic load was measured.

Parasite strains

L. amazonensis promastigotes (PH8) were grown in medium 199 supplemented with 20% FCS, 0.1 mM adenine (Life Technologies, Gaithersburg, MD), 25 mM HEPES (Life Technologies), 5 g/ml hemin (Sigma-Aldrich, St. Louis, MO), 1 g/ml biotin (Life Technologies), and Pen/Strep/L-glutamine (Life Technologies). *L. major* clone V1 promastigotes (MHOM/IL/80/Friedlin) were grown at 26°C in the same medium. Infective-stage metacyclic *L. major* promastigotes were isolated from 4- to 5-day-old stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA), whereas infectious *L. amazonensis* promastigotes were purified by negative selection using mAb D5, as previously described (28, 30). In challenge experiments, 10^7 purified metacyclic promastigotes suspended in RPMI 1640 were injected i.d. into the forehead of macaques (15).

Parasite load

Parasite load was estimated as described (28). Briefly, 2-mm^2 biopsies were taken, treated with 1 mg/ml collagenase A (Sigma-Aldrich) for 2 h at 37°C, homogenized, filtered, and serially diluted in a 96-well flat-bottom microtiter plate containing biphasic medium, prepared using 50 μl of NNN medium containing 30% defibrinated rabbit blood and overlaid with 50 μl of M199/S. The number of viable parasites in each lesion was determined from the highest dilution at which promastigotes could be grown out after 7 days of incubation at 26°C. The total number of parasites in the lesion was obtained by multiplying the number of parasites in the biopsy by the area of the lesion.

Antibodies

Ab pairs that recognize both human and macaque IL-6 (R&D Systems, Minneapolis, MN), and IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ) were used in ELISA. Abs specific for human (Endogen, Woburn, MA) or macaque (Bender MedSystems, Vienna, Austria; Mabtech, Stockholm, Sweden) were used to measure IFN-γ.

ELISA

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with anti-cytokine Ab and blocked with PBS-5% BSA (12). Culture supernatants were added, and their cytokine content was quantitated by the addition of biotin-labeled anti-cytokine Ab followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human cytokine. All assays were performed in triplicate. When supernatants from

Table I. *Characteristics of HIV-infected PBMC donors*

	<200 CD4$^+$ T Cells	200–500 CD4$^+$ T Cells	>500 CD4$^+$ T Cells
n	9	17	17
Age	40 ± 2	39 ± 1	37 ± 2
Race (white/black/Hispanic)	4/4/1	13/4/1	8/7/2
Gender (male/female)	8/0	15/2	17/0
CD4$^+$ T Cells	25 ± 7	317 ± 20	735 ± 67
% CD4$^+$ T Cells	3 ± 1	21 ± 1.9	31 ± 3
Viral load	27,000 ± 50,000	1,828 ± 29,000	663 ± 330
Viral load range	ND–75,000	ND–500,000	ND–35,000
% CD56$^+$/CD16 cells	9 ± 2	8.3 ± 1	5.6 ± 1.6
% CD19$^+$ cells	19.5 ± 5	14 ± 1	9 ± 2
% CD14$^+$ cells	19 ± 2	22 ± 1	15.6 ± 3
% on HAART	66	66	80

HIV/SIV-infected PBMCs were used, 0.02% Triton X-100 was added to the washing buffer to inactivate the virus.

Cell proliferation assay

A total of 10^5 PBMCs/well were incubated with 1–3 μM ODN for 68 h, pulsed with 1 μCi of [^{3}H]thymidine, and harvested 4 h later. All assays were performed in triplicate. Intraassay variation was <15%.

Flow cytometry

Cultured cells were washed in cold PBS, fixed, and stained with fluorescent-labeled Abs to CD4, CD56, CD16, CD19, B220, CD83, CD86, CD14, and MHC class II as previously described (18). Samples were washed and analyzed (20,000–40,000 events) on a FACScan flow cytometer (BD Biosciences, San Jose, CA). The number of DCs was obtained after gating on monocytes with proper electronic compensation. The data were analyzed with CellQuest software (BD Biosciences).

Viral load measurements

SIV plasma RNA levels were determined by a real-time RT-PCR assay, as described (31).

Statistical analysis

Statistically significant differences in cytokine and cell proliferation levels were determined using a two-tailed nonparametric rank sum test or ANOVA with Dunnett's post-test analysis. Spearmann's correlations were used to assess the relationship between viral load or number of CD4 T cells and response to ODNs. Differences in lesion sizes were tested by Friedman Repeated-Measures Analysis on Ranks with Tukey's All Pairwise Multiple Comparison Procedure using Sigma Stat (SPSS, San Rafael, CA). Differences in parasite load were tested by t test of log-normalized data.

Results

PBMCs from normal and HIV-infected donors respond to CpG ODNs

Retrovirus infection is associated with a progressive loss of immune function and increased susceptibility to opportunistic infections. CpG ODNs that activate PBMCs from normal human donors (14) were assessed for their ability to stimulate cells of the innate immune system of HIV patients. Consistent with previous reports, K ODNs preferentially induced cell proliferation and IL-6 production in PBMCs from healthy subjects, whereas D-type ODNs stimulated the secretion of IFN-α and IFN-γ (Fig. 1). As reported previously, pure phosphorothioate ODNs (non-CpG K-type controls) induced low-level, sequence-nonspecific cell proliferation. This phosphorothioate-dependent activation was significantly lower than that elicited by K ODN ($p < 0.05$) (5, 32). In addition, D ODNs, but not K ODNs, triggered the maturation of DCs in vitro, as characterized by increased expression of CD83 and CD86 (Fig. 2) (18).

PBMCs from HIV-infected and healthy subjects responded similarly to K-type ODNs (Fig. 1), suggesting that B cells and monocytes retained their ability to respond to this form of immune stimulation. Although D-type ODNs induced a significant increase in cytokine secretion by cells from both donor populations ($p < 0.001$), the IFN-α and IFN-γ response of healthy controls significantly exceeded that of HIV-infected subjects ($p < 0.05$ and $p < 0.001$, respectively; Fig. 1). This reduced responsiveness to D ODNs correlated directly with the number of CD4$^+$ T cells among the HIV-infected donors ($p < 0.01$; Fig. 3) and inversely with their viral load ($p < 0.05$; data not shown). No significant correlation between cytokine production and the number of CD56$^+$ NK cells or CD14$^+$ monocytes was observed (data not shown). D ODNs also maintained their ability to trigger the maturation of DCs from HIV-infected donors. As seen in the examples in Fig. 2, the absolute number of mature DCs was lower in the unstimulated PBMCs from HIV-infected donors than in normal donors (0.13 ± 0.06% vs 0.26 ± 0.07%, respectively; $p < 0.05$; data not shown). However, treatment with D ODNs increased the number of CD83$^+$CD86$^+$ cells by ~20-fold (to 2 ± 1% vs 6 ± 1%, respectively; data not shown) in both groups.

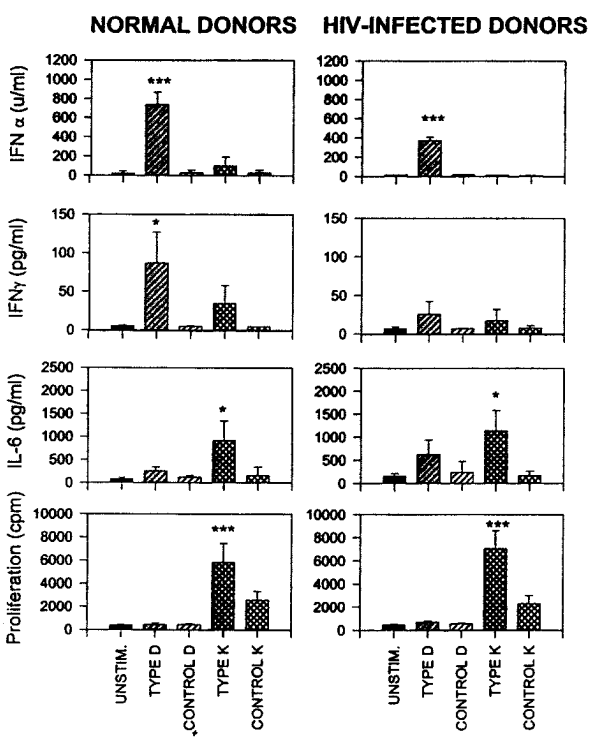

FIGURE 1. Response of human PBMCs to K and D ODNs. PBMCs from 16 healthy blood donors and 43 HIV-infected subjects were stimulated for 72 h with optimal concentrations of K3 (1 μM), D29 (3 μM), control K163 (1 μM), or control D122 (3 μM). IFN-α, IFN-γ, and IL-6 levels in culture supernatants were determined by ELISA, whereas cell proliferation was assessed by [^{3}H]thymidine uptake. Note that D ODNs induce the secretion of IFN-α and IFN-γ, whereas K ODNs induce higher cell proliferation and IL-6 production. All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. *, $p < 0.05$; ***, $p < 0.001$.

PBMCs from normal and SIV-infected macaques respond to CpG ODNs

Rhesus macaques provide a useful model for evaluating the activity of CpG ODNs planned for human use (15, 16, 33, 34). Previous studies established that PBMCs from these animals respond to the same D and K ODNs that activate human PBMCs (15). We compared the responses of PBMCs from 16 immunocompromised SIV-infected animals to those of 20 healthy macaques. Consistent with results involving PBMCs from HIV-infected patients, PBMCs from SIV-infected macaques responded normally to K ODNs in vitro (Fig. 4). Their IFN-α response to D ODNs, by comparison, was significantly reduced when compared with PBMCs from healthy controls ($p < 0.01$; Fig. 4). Moreover, although healthy macaques responded to D ODNs by secreting IFN-γ, no detectable IFN-γ was detectable in PBMCs from SIV-infected macaques.

Immunoprotective activity of CpG ODNs in healthy macaques

Previous studies established that CpG ODNs can decrease the magnitude and duration of *Leishmania* infection in mice (8, 10, 35). A self-limiting cutaneous *L. amazonensis* challenge model was used to evaluate whether CpG ODNs could similarly protect rhesus macaques (26). Macaques were injected i.d. on days −3 and 3 with 500 μg of CpG ODNs that activate PBMCs from human

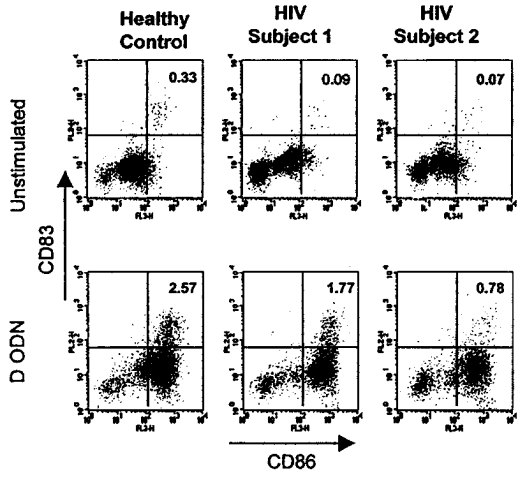

FIGURE 2. D ODNs induce monocytes to differentiate into mature DCs. Mature DCs ($CD83^+CD86^+$) were identified by FACS analysis of PBMCs from healthy and HIV-infected subjects. Note that the number of mature DCs in samples from HIV-infected patients increases 10- to 20-fold after 72 h of culture with 3 μM D ODN. Two representative examples of six experiments are shown.

and nonhuman primates (15). On day 0, the animals were challenged at the same site (forehead) with 10^7 metacyclic *L. amazonensis* promastigotes. Naive animals developed a cutaneous lesion similar to those found in human cutaneous leishmaniasis (26), with a peak surface area of 4.4 ± 0.7 mm^2 on day 22 (Fig. 5). Lesion size was significantly reduced among macaques treated with D-type ODN ($p < 0.001$; Fig. 5). In contrast, the severity of *Leishmania* infection in animals treated with K ODN was not significantly different from that of the controls ($p = 0.1$).

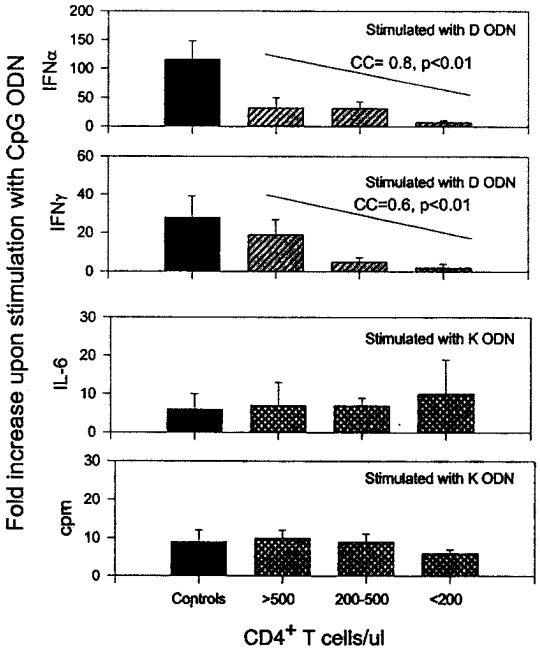

FIGURE 3. Relationship between $CD4^+$ T cell count and CpG ODN responsiveness. The response of PBMCs from HIV-infected donors (n = 42) to D (*upper two panels*) and K (*lower two panels*) ODNs was stratified by CD4 T cell count. Data show the fold increase in cytokine production and proliferation of treated vs unstimulated cells. The correlation coeficient (CC) and the p value were obtained using Spearman's Correlations.

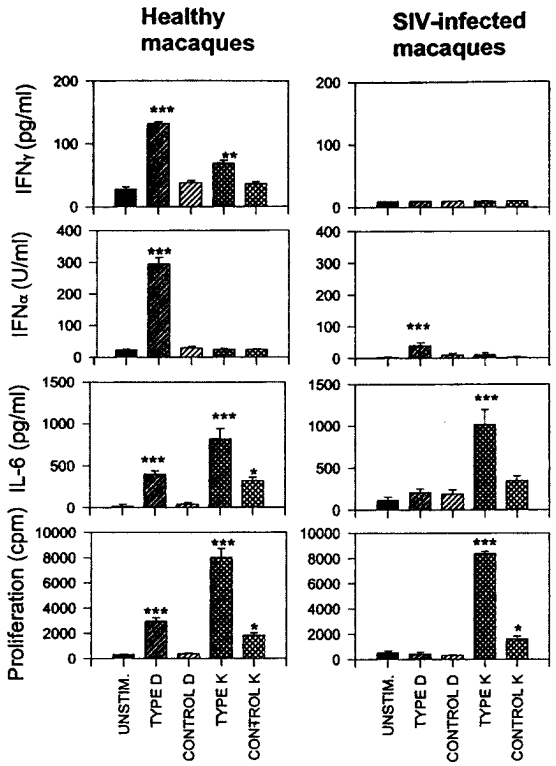

FIGURE 4. Response of PBMCs from SIV-infected and healthy rhesus macaques to CpG ODNs in vitro. PBMCs from 16 SIV-infected and 20 healthy macaques were stimulated for 72 h with K, D, or control ODN. IFN-γ, IFN-α, and IL-6 levels in culture supernatants were determined by ELISA, whereas cell proliferation was assessed by [^{3}H]thymidine uptake. The detection limit for the assays was 20 pg/ml for IFN-γ, IFN-α, and IL-6. All assays were performed in triplicate. Statistical significance was determined by a one-way ANOVA of log normalized data. *, $p < 0.05$; ***, $p < 0.001$.

Immunoprotective activity of CpG ODNs in SIV-infected macaques

Based on the observation that CpG ODNs retain the ability to activate PBMCs from retrovirus-infected primates, their ability to reduce the severity of a *Leishmania* infection in SIV-infected macaques was examined. Macaques that had been infected >12 mo earlier with SIVmac239 and that had viral loads ranging from 0.3 to 28 × 10^6 copies/ml were used in this study. The animals were stratified based on viral load and then were challenged with *L. major* metacyclic promastigotes (MHOM/IL/80/Friedlin). As shown previously, healthy macaques challenged with *L. major* developed cutaneous lesions characterized by erythema, induration, and ulceration that peaked 25 days after challenge and resolved within 50 days (Fig. 6*A* and Ref. 15). Due to their immunosuppressed state, the macaques developed severe progressive cutaneous lesions that did not resolve. The severity of *Leishmania* infection in animals treated with K ODNs was not significantly different from that of the controls. In contrast, macaques treated with D ODNs developed significantly smaller lesions, and their infection did not progress over time (Fig. 6*A*).

Animals were euthanized on day 56, and their parasite burden was measured. Monkeys treated with D ODNs had a 35-fold reduction in total parasite burden at the lesion site compared with SIV-infected animals treated with control ODNs or saline (Fig. 6*B*; $p < 0.001$). No systemic spread of the parasites was evident in any of the groups.

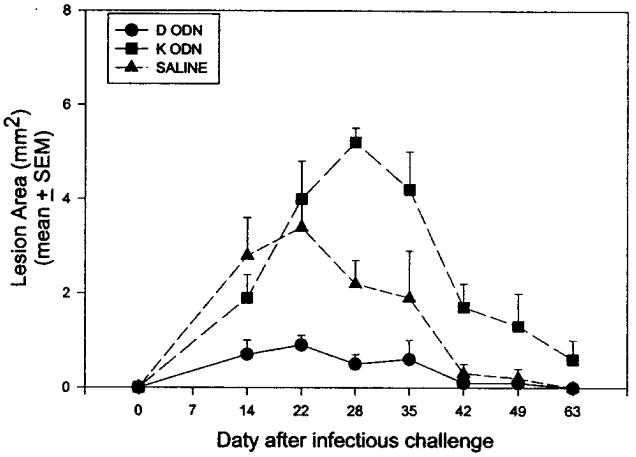

FIGURE 5. Effect of CpG ODN treatment on cutaneous leishmaniasis. Macaques (six per group) were treated with 500 μg of a mixture of D ODN, K ODN, or saline i.d. 3 days before and 3 days after an infectious challenge with 10^7 *L. amazonensis* metacyclic promastigotes. The mean and SEM of the area of the lesions is shown. Note that macaques treated with D ODN had significantly smaller lesions ($p < 0.05$).

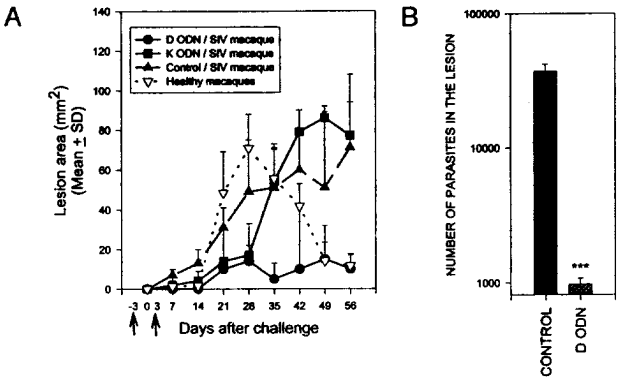

FIGURE 6. Effect of CpG ODN treatment on *Leishmania* lesions in SIV-infected monkeys. Macaques infected for >12 mo with SIVmac239 were treated with 250 μg of a mixture of D ($n = 4$) or K ($n = 4$) ODN i.d. 3 days before and 3 days after an infectious challenge with 10^7 *L. major* metacyclic promastigotes. Controls include untreated healthy macaques ($n = 6$) and SIV-infected macaques treated with either control ODN ($n = 3$) or saline ($n = 3$). *A*, Mean area of the lesions. *B*, Estimated total parasite load on day 56 in control vs D ODN-treated, SIV-infected macaques. Note that macaques treated with D ODN had significantly smaller lesions ($p < 0.05$) as well as lower parasite loads ($p < 0.001$).

Concurrent *Leishmania* infection can activate the HIV present in latently infected monocytes and T cells, thereby increasing viremia (36). Therefore, viral load measurements were conducted in *Leishmania*-infected macaques every 2 wk throughout the study. No significant change in viral load was evident in any of the groups (data not shown).

Discussion

CpG ODNs stimulate the innate immune system, thereby improving the host's resistance to infectious pathogens. Previous studies established that mice treated with CpG ODNs could survive otherwise lethal infections by *Listeria, Franciscella*, and *Leishmania* (8–11). Yet the CpG motifs that are highly active in rodents are poorly immunostimulatory in humans, limiting the utility of murine models to examine whether CpG ODNs can protect primates such as humans (12, 15). This study establishes that K and D CpG ODNs induce PBMCs from both normal and immunosuppressed primates to mature, proliferate, and secrete cytokines. Moreover, it demonstrates that CpG ODNs enhance the ability of both normal and immunosuppressed primates to resist pathogen challenge.

HIV infection results in not only a progressive reduction in $CD4^+$ T cells, but also a decrease in the number and functional activity of NK cells and plasmacytoid DCs as viral load rises (20, 21, 37, 38). Previous studies established that CpG ODNs activated PBMCs from normal donors; the present work extends that work to HIV-infected subjects. Although the number of mature DCs in the peripheral blood of HIV-infected donors is reduced (Fig. 2 and Refs. 21 and 39), PBMCs from retrovirus-infected humans and macaques responded to both D and K ODNs. Indeed, the magnitude of the response to K ODNs was essentially unaffected by retroviral infection, although the response to D ODNs was reduced in HIV- and SIV-infected donors. It is unlikely that the changes in responsiveness to D ODNs observed in PBMCs from HIV-infected donors were related to their antiretroviral therapy because 1) no significant correlation between the CpG response and antiretroviral therapy was evident and 2) a similar reduction in the response to D ODNs was evident in untreated SIV-infected monkeys. Despite the decline in IFN-γ and IFN-α response to D ODNs in SIV-infected macaques, the immune activation induced by these agents was sufficient to control a superinfection with *Leishmania*.

Murine studies established that protection against this parasite correlated with the production of type 1 cytokines, particularly IL-12 (40). It was unclear whether CpG ODNs could induce protection against *Leishmania* in primates because 1) primates and rodents respond optimally to different CpG motifs (12, 14) and 2) primates fail to produce large amounts of IL-12 when treated with CpG ODNs (41). Cutaneous infection of macaques with *Leishmania* provided a means for examining this question, because the nature, severity, and duration of this infection in macaques and humans is quite similar (26), and PBMCs from these species respond to the same CpG motifs (Figs. 1 and 4 and Refs. 15 and 28). As seen in Fig. 5, normal macaques treated with D ODNs developed significantly smaller lesions than control animals or animals treated with K ODNs after *L. amazonensis* infection. D ODN treatment of immunosuppressed SIV-infected monkeys also yielded protection against cutaneous leishmaniasis, despite their inability to induce IFN-γ, as reflected by smaller lesions and reduced parasite load (Fig. 6). Although viceral leishmaniasis rather than cutaneous leishmaniasis is of greatest concern in HIV patients, the reduced lesion size in these animals suggests that CpG treatment may contribute to the control of intracellular infections in these patients.

Because D ODNs excel at stimulating the production of Th1 cytokines and type 1 cytokines inhibit parasite proliferation, it is not surprising that D ODNs were the most effective at reducing the pathogenic effects of *Leishmania* infection (8, 42). In contrast, K ODNs neither stimulated Th1 cytokine production nor had any significant effect on the onset, magnitude, or duration of the *Leishmania* infection (8, 14, 17, 18). It is likely that functional differences between D- and K-type ODNs are due to differences in their structures. K ODNs have a phosphorothioate backbone and optimally contain multiple TCGTT and/or TCGTA motifs. D ODNs have a mixed phosphodiester/phosphorothioate backbone, contain a single self-complementary purine/pyrimidine/CpG/purine/pyrimidine motif, and are capped by a 3′ poly G tail (14). Ongoing studies suggest that these structural differences are associated with differences in the recognition, uptake, and/or processing of these two types of ODN by immune cells (43).

Over 30 million people are currently infected with HIV worldwide (44). Recent studies indicate that HIV patients are more susceptible to leishmaniasis (an estimated 9% of AIDS patients are co-infected with *Leishmania*) (45). In addition to the compromised immune status, HIV infection has been shown to enhance the intracellular growth of *Leishmania* in macrophages (46), which may explain why HIV patients tend to develop more aggressive visceral forms of that disease (45, 46). *Leishmanias*, in turn, can increase HIV viral load by activating latently infected monocytes and inducing chronic T cell activation (36). Current studies demonstrate that type D ODNs can reduce the severity of *Leishmania* infections by 35-fold in immunosuppressed subjects. The persistence of lesions even after D ODN treatment suggests that a combination of CpG ODNs with other antiparasitic agents may be required to cure this disease. Testing the efficacy of such combinations in both cutaneous and visceral models of leishmaniasis is an important goal of future research. Current results document that inducing a strong innate immune response can reduce host susceptibility to infection. Thus, CpG treatment may benefit normal individuals at increased risk of environmental exposure to infectious agents and may help to reduce the morbidity and mortality of opportunistic infections among the immunosuppressed. As such, CpG ODNs (alone or in combination with other agents) may become a valuable addition to conventional antiretroviral therapy.

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CpG Oligodeoxynucleotides as Vaccine Adjuvants in Primates[1]

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Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants in mice, boosting the humoral and cellular response to coadministered Ags. CpG ODN that stimulate human PBMC are only weakly active in mice. Thus, alternative animal models are needed to monitor the activity and safety of "human" CpG ODN in vivo. This work demonstrates that rhesus macaques recognize and respond to the same CpG motifs that trigger human immune cells. Coadministering CpG ODN with heat-killed *Leishmania* vaccine provided significantly increased protection of macaques against cutaneous *Leishmania* infection. These findings indicate that rhesus macaques provide a useful model for studying the in vivo activity of human CpG motifs, and that ODN expressing these motifs act as strong immune adjuvants. ***The Journal of Immunology,*** **2002, 168: 1659–1663.**

Synthetic oligodeoxynucleotides (ODN)[4] containing unmethylated "CpG motifs" are broadly immunostimulatory in mice (1–4). They activate B cells and dendritic cells (DC), and trigger an immune cascade that includes the production of cytokines, chemokines, and IgM (1, 2, 5–8). In mice, CpG ODN boost the protective efficacy of vaccines against bacterial, viral, and parasitic pathogens (9–14).

Due to evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents are poorly immunostimulatory in primates, and vice versa (15–17). Extensive studies involving human PBMC identified two distinct classes of immunostimulatory CpG ODN (17, 18). "K" type ODN have phosphorothioate backbones, encode multiple TCGTT and/or TCGTA motifs (CpG motif is underlined), trigger the maturation of plasmacytoid DC, and stimulate the production of IgM and IL-6 (17, 19). "D" ODN have mixed phosphodiester/phosphorothioate backbones and contain a single hexameric purine/pyrimidine/CG/purine/pyrimidine motif flanked by self-complementary bases that form a stem-loop structure capped at the 3′ end by a poly G tail (17). D ODN trigger the maturation of APC and preferentially induce the secretion of IFN-α and IFN-γ (Refs. 17 and 18 and M. Gursel, unpublished observations).

There is considerable interest in evaluating the safety and activity of CpG ODN planned for human use in a relevant animal model. Although Davis and colleagues (20–22) showed that K ODN increased the seroconversion rate and Ab response of orangutans and aotus monkeys immunized with hepatitis B vaccine and malaria proteins, that group was unable to document improved protection against infection by challenge studies. Moreover, no studies have compared the activity of K ODN with the recently discovered D class of ODN in primates.

This work examines whether rhesus macaques provide a useful model for assessing the activity of CpG ODN in vivo. In vitro studies established that PBMC from rhesus macaques responded to the same panel of K and D ODN that were highly active on human PBMC. Building on results from murine studies (23, 24), CpG ODN were coadministered with a mixture of OVA plus alum. The ODN significantly boosted the Ag-specific IgG response of macaques, with D being superior to K ODN. A cutaneous *Leishmania* infection model was used to examine whether CpG ODN could boost protective immunity in primates. The nature, severity, duration, and histopathology of the cutaneous disease caused by *Leishmania major* in macaques is quite similar to that in humans (25, 26). Results indicate that D ODN significantly improve the protection conferred by coadministered heat-killed *Leishmania* vaccine (HKLV).

Materials and Methods

Rhesus monkeys

Healthy 3-year-old female rhesus macaques (*Macaca mulatta*) were obtained from the Food and Drug Administration colony in Morgan Island, SC. All studies were approved by the Center for Biologics Evaluation and Research Animal Care and Use Committee, and were conducted in an American Association for the Accreditation of Laboratory Animal Care-accredited facility. Animals were monitored daily by veterinarians. No systemic or local adverse reactions to CpG ODN, OVA, or HKLV immunizations were observed. Treatments were administered and peripheral blood samples obtained from ketamine-anesthetized animals (10 mg/kg, Ketaject; Phoenix Pharmaceuticals, St. Joseph, MD).

Vaccination groups and protocol

Two in vivo studies were conducted: 1) three monkeys per group were immunized s.c. and boosted 12 wk later with a mixture of 4 μg of OVA, 250 μg of ODN, and 125 μg of alum (Rehydragel HPA; Reheis, Berkeley Heights, NJ); 2) five to six monkeys per group were immunized s.c., and boosted 4 wk later with 250 μg of GMP-grade HKLV (Biobras, Montes Claros, Brazil) plus 125 μg of alum, as previously described (27). The HKLV was administered alone, or combined with 500 μg of ODN. Preliminary studies established that this dose of ODN was active in vivo and well-tolerated. Animals were exposed to nonviable *Leishmania amazonensis* metacycle promastigotes on wk 8, a treatment that induced no disease

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[4] Abbreviations used in this paper: ODN, oligodeoxynucleotide; SLA, soluble *Leishmania* Ag; DC, dendritic cell; HKLV, heat-killed *Leishmania* vaccine.

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and no change in Ab titer or proliferative response to *Leishmania* Ags when compared with control animals. Animals were challenged on the forehead on wk 14 with 10^7 viable *L. major* (WHOM/IR/-/173) metacyclic promastigotes intradermally. The monkeys developed a typical self-limited in situ lesion characterized by erythema, induration, and ulceration. Lesion size, which reflects the severity of infection (25, 26), was measured weekly.

Oligodeoxynucleotides

ODN (Table I) were synthesized by the Center for Biologics Evaluation and Research Core Facility. All ODN had <0.1 EU of endotoxin per milligram of ODN as assessed by a *Limulus* amebocyte lysate assay (QCL-1000; BioWhittaker, Gaithersburg, MD).

Mononuclear cell preparation

Human and monkey mononuclear cells were isolated by density gradient centrifugation of PBMC over Ficoll-Hypaque as described (17). Cells were washed three times and cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1.5 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 5×10^5 cells/well in the presence of 3 μM ODN. Supernatants were collected after 72 h and tested by ELISA for cytokine and Ab levels.

ELISA

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with Abs that cross-reactively recognized human and macaque IL-6 (R&D Systems, Minneapolis, MN), IFN-α (PBL Biomedical Laboratories, New Brunswick, NJ), and IgG (Boehringer Mannheim, Mannheim, Germany). The plates were blocked with PBS-5% BSA (17). Culture supernatants from PBMC cultures were added, and their cytokine content quantitated by the addition of biotin-labeled anti-cytokine Ab followed by phosphatase-conjugated avidin and phosphatase-specific colorimetric substrate. Standard curves were generated using known amounts of recombinant human cytokine or purified Ig. All assays were performed in triplicate. Titers of Abs to OVA in sera were assayed on OVA-coated plates.

ELISPOT

The number of PBMC secreting IFN-γ in response to soluble *Leishmania* Ag (SLA) was determined by ELISPOT as described (28). Briefly, Millipore 96-well filtration plates (Millipore, Bedford, MA) were coated overnight at 4°C with 1 μg/ml of anti-human IFN-γ Abs (clone GZ4; Alexis, San Diego, CA) in PBS and then blocked with PBS-5% BSA for 2 h. The plates were overlaid with 5×10^5 cells/well (1–2 series per monkey) and incubated at 37°C in a humidified 5% CO_2 in air incubator for 18 h in the presence of 25 μg of SLA. The plates were then washed with water-0.025% Tween and overlaid with biotin-conjugated anti-human IFN-γ (clone 76-B-1; Mabtech, Nacka, Sweden). After 2 h, the plates were washed again and then overlaid with alkaline phosphatase-conjugated streptavidin. Spots were visualized by the addition of 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and counted using the KS ELISPOT Imagine System (Carl Zeiss, Thornwood, NY).

Cell proliferation assay

A total of 10^5 PBMC/well were incubated with 3 μM of ODN for 68 h, pulsed with 1 μCi of [^{3}H]thymidine and harvested 4 h later. All assays were performed in triplicate.

Statistical analysis

Statistically significant differences were determined using a two-tailed nonparametric ANOVA with Dunnett's post test analysis. Differences in lesion sizes were tested by repeated measures ANOVA using the Proc Mixed procedure from the statistical analysis system.

Table I. *Sequence and backbone of D, K, and control ODN*[a]

ODN	Sequences
D19	GG**TGCATCGATGCAGG**GGGG
D29	GG**TGCACCGGTGCAGG**GGGG
D35	GG**TGCATCGATGCAGGGG**GG
D122	GG**TGCATTGATGCAGG**GGGG
K3	ATCGACTCTCGAGCGTTCTC
K123	TCGTTCGTTCTC
K23	TCGAGCGTTCTC
K163	TTGAGTGTTCTC
AA3M	GG**GCATGCATGG**GGGG

[a] CpG dinucleotides are underlined. Bases in bold are phosphodiester.

Results

Response of PBMC from human and nonhuman primates to K and D ODN

Previous studies established that human PBMC respond to two structurally distinct classes of CpG ODN (17). D-type ODN triggered the secretion of IFN-α and IFN-γ (17), whereas K ODN induced human PBMC to proliferate and secrete IL-6 and IgM (Fig. 1, Ref. 17, and data not shown). Analysis of several hundred CpG ODN identified several D and K ODN that strongly activated human PBMC (17). These ODN were tested for their ability to stimulate PBMC from rhesus macaques.

In this study, the response of rhesus PBMC to D ODN was evaluated on the basis of IFN-α production. Results show that macaque PBMC are activated by the same D ODN that stimulate human PBMC ($p < 0.002$, Fig. 1). In contrast, neither K ODN, nor control ODN that are structurally similar to D but lack the critical CpG dinucleotide have this effect.

Proliferation and IL-6 secretion were used to compare the response of macaque and human PBMC to K ODN (Fig. 1). PBMC from both species were stimulated by K ODN to proliferate ($p < 0.002$) and secrete IL-6 ($p < 0.01$), whereas controls of the same structure as K ODN, but lacking the critical CpG motif, failed to trigger immune stimulation. These findings demonstrate that the pattern of reactivity of PBMC from rhesus macaques ($n = 20$) and humans ($n = 8$–20) to K and D ODN is quite similar.

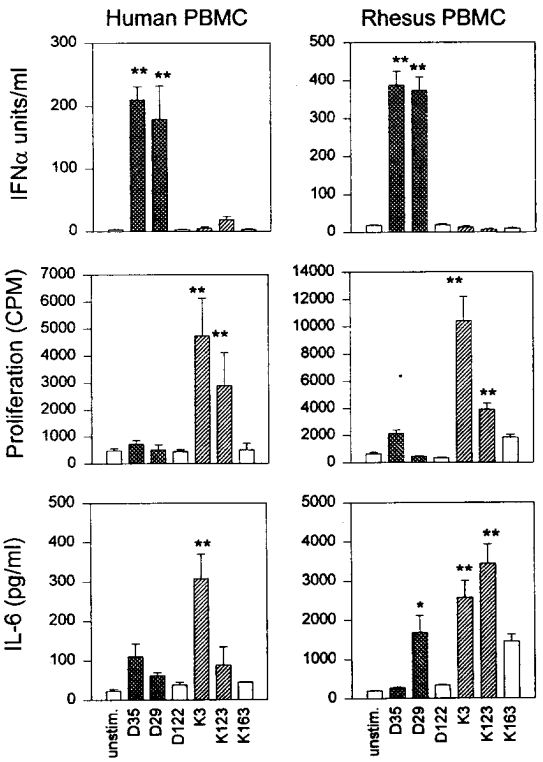

FIGURE 1. Response of primate PBMC to K and D ODN. PBMC from 8–20 normal human donors and 20 rhesus macaques were stimulated for 72 h with 3 μM of K, D, or control ODN (in which the critical CpG motifs were inverted or replaced with TpG). IL-6 and IFN-α levels in culture supernatants were determined by ELISA, while cell proliferation was assessed by [^{3}H]thymidine uptake. Note that D ODN induce the secretion of IFN-α while K ODN induce cell proliferation and IL-6 production. All assays were performed in triplicate. Statistical significance was determined by ANOVA of log normalized data. *, $p < 0.05$; **, $p < 0.01$.

Ongoing studies in our lab indicate that individual humans and monkeys vary in their response to specific K and D sequences. Indeed, no single D or K motif is optimally stimulatory in all donors (Ref. 29 and C. Leifer, unpublished observations). However, mixtures of ODN were identified that strongly stimulated PBMC from all human donors. These mixtures were tested on PBMC from macaques and found to be uniformly active (Fig. 2). Subsequent in vivo studies were conducted with these ODN mixtures.

Adjuvant activity of CpG ODN in vivo

Previous studies in mice showed that CpG ODN could boost the immune response to a coadministered protein Ag (such as OVA). This effect was amplified by adding alum to the mixture of CpG ODN plus Ag (23, 30, 31). Building on these results, macaques were immunized and boosted with a mixture of OVA, alum, and ODN. Animals immunized with mixtures containing D ODN increased their IgG anti-OVA response 470-fold after primary ($p < 0.05$) and 600-fold after secondary ($p < 0.01$) immunization (Fig. 3). By comparison, K ODN boosted the IgG Ab response 7-fold after primary, and 35-fold after secondary immunization when compared with pretreatment values ($p < 0.05$). Macaques immunized with OVA plus control ODN generated only a 4-fold increase in anti-OVA titer. These findings indicate that D ODN are particularly effective at boosting the Ag-specific humoral response to a coadministered Ag.

Effect of CpG ODN on the immunogenicity and protective efficacy of HKLV

Previous human clinical trials showed that HKLV was safe, but poorly immunogenic (26). Building on evidence that HKLV combined with alum and IL-12 induces short-term protective immunity in rhesus macaques (27), and that CpG ODN plus alum increased the immune response to the hepatitis B vaccine in cyalomongus monkeys (20), we immunized and boosted macaques with a mixture of HKLV, alum, and CpG ODN. PBMC from these animals were isolated 10 days postboost and restimulated in vitro with

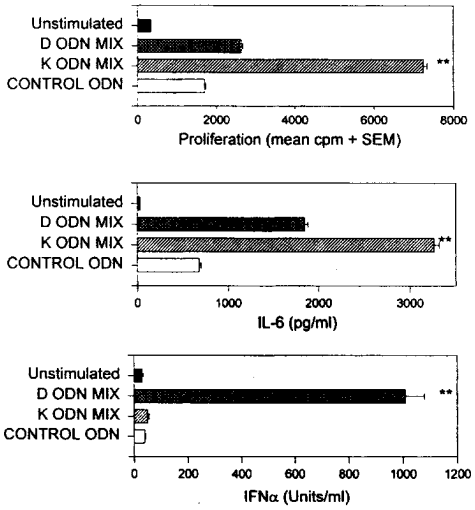

FIGURE 2. Macaque PBMC respond to CpG ODN mixtures optimized for human use. PBMC from rhesus macaques (n = 12–20) were stimulated in vitro for 72 h with a mixture of D19, D29, and D35 (1 μM each) or K3 and K123 (1.5 μM each). D122 and K163 were used in the control ODN mixture. Levels of IL-6 and IFN-α in culture supernatants were measured by ELISA, while proliferation was measured by [^{3}H]thymidine uptake. Statistical significance was determined by ANOVA of the normalized data. **, $p < 0.01$.

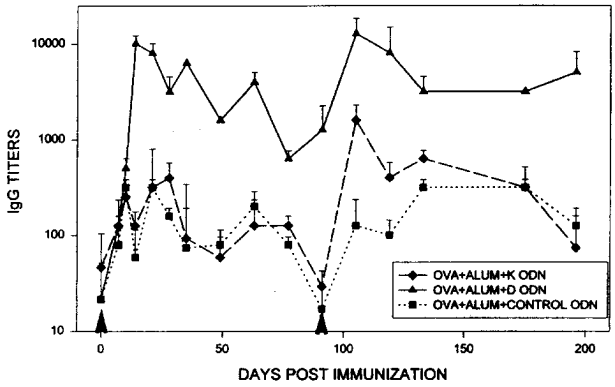

FIGURE 3. Antibody titers in macaques immunized with OVA plus ODN. Macaques (three per group) were immunized s.c. with a mixture of 4 μg of OVA plus 125 μg of alum. A total of 250 μg of D19 + D29, K3 + K23, or control (AA3 M) ODN was added to this mixture. Monkeys were boosted with the same material 12 wk later (black arrow). Serum IgG anti-OVA titers were determined by ELISA. Values represent the geometric mean titer ± SEM. Note that the anti-OVA IgG titers in the group that received D ODN are significantly increased over that of OVA plus alum alone ($p < 0.01$).

Leishmania Ag for 18 h. As seen in Fig. 4, both K and D ODN significantly increased the number of PBMC triggered to secrete IFN-γ ($p < 0.05$). In contrast, animals immunized with alum-adsorbed HKLV alone showed no increased IFN-γ production when compared with unimmunized controls.

The critical measure of an Ag/adjuvant combination is its ability to induce protective immunity. Vaccinated animals were therefore challenged with 10^7 *L. major* metacyclic promastigotes. Animals vaccinated with HKLV-alum alone developed typical cutaneous lesions with a peak surface area of 300 ± 60 mm^2 26 days after challenge (Fig. 5). Monkeys vaccinated with HKLV-alum plus K ODN developed lesions of similar size, although the peak lesion formation was slightly delayed. Animals immunized with HKLV-alum plus D ODN had significantly smaller lesions (maximal size 80 ± 13 mm^2, $p < 0.05$), consistent with a reduced parasite burden (32).

CpG ODN safety

All animals treated with CpG ODN, either alone or with Ag, remained healthy and active throughout the study. No hematologic

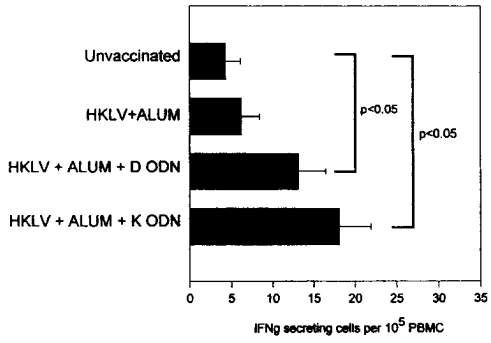

FIGURE 4. IFN-γ production by PBMC from macaques immunized with alum-adjuvanted HKLV plus ODN. Rhesus macaques were immunized and boosted with 250 μg of HKLV-alum alone (n = 6), or combined with 500 μg of a mixture of D (D19, D29, and D35; n = 5) or K (K3 and K123; n = 5) ODN. PBMC from these animals were incubated with 25 μg of SLA and analyzed in vitro for IFN-γ production by ELISPOT assay. Animals immunized with HKLV plus K or D ODN had significantly more IFN-γ-secreting cells than unvaccinated controls as determined by a one-way ANOVA ($p < 0.05$).

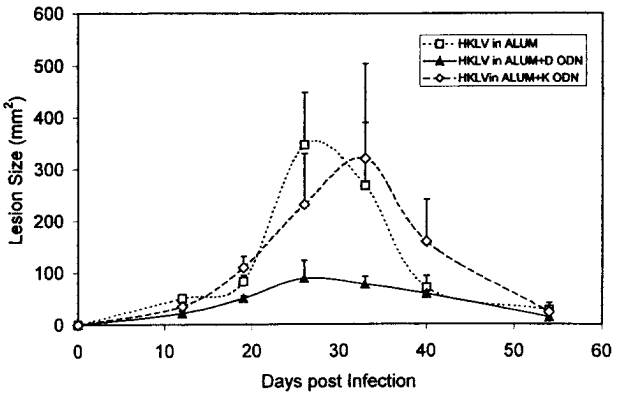

FIGURE 5. Cutaneous lesions in macaques vaccinated with alum-adjuvanted HKLV plus ODN. Rhesus macaques were primed s.c. with 250 μg of alum-adjuvanted HKLV alone ($n = 6$) or combined with 500 μg of a mixture of ODN (D19, D29, and D35; $n = 5$) or (K3 and K123; $n = 5$) and boosted 4 wk later. On wk 14, the monkeys were challenged with 10^7 metacyclic promastigotes. The average size of the lesions on the forehead (the site of challenge) is shown as the mean area (calculated as mean diameter/2 $\times$ π). Note that macaques immunized with HKLV plus D ODN had significantly smaller lesions ($p < 0.01$).

or serologic abnormalities were observed 3 days or 9 mo after injection, and no weight loss or change in behavior was detected following administration of CpG ODN at therapeutic doses.

Discussion

CpG ODN that activate human immune cells in vitro are only weakly immunostimulatory in mice. To expedite preclinical testing of the safety and activity of human ODN requires the identification of a suitable animal model. This report documents that rhesus macaques provide a relevant model for examining the in vivo activity of CpG ODN planned for human use. PBMC from macaques mirrored the response of human PBMC in their response to both K and D ODN. At the immunostimulatory doses used in this study, neither type of ODN triggered any adverse events. In vivo, broadly immunostimulatory mixtures of K and D ODN boosted Ag-specific IgG responses in macaques immunized with OVA and increased IFN-γ production in animals vaccinated with HKLV. Of greater importance, D ODN significantly increased the protective response elicited by a coadministered HKLV vaccine.

Several previous reports examined whether K ODN could act as immune adjuvants in nonhuman primates (20–22). Studies by Davis and colleagues (20–22) demonstrated that K ODN boosted the Ag-specific serum IgG response to alum-adjuvanted hepatitis B vaccine, and to a peptide from the circumsporozoite protein of malaria in orangutans and aotus monkeys. This was consistent with results from earlier studies in mice showing that CpG ODN plus alum synergize to boost immunity to Ag (23, 30, 31, 33). Yet these experiments did not establish whether the resulting immune response conferred protection against infection. The current experiments confirm that K ODN boost the Ab response to a coadministered protein (OVA). They further document that D ODN are significantly more effective in this role, boosting Ab production by >500-fold over pretreatment levels and >100-fold over OVA plus alum (Fig. 3).

Cutaneous infection of macaques with *L. major* provides a means for testing the protective efficacy of CpG ODN vaccine combinations. The nature, severity, and duration of the cutaneous disease caused by *L. major* in macaques is quite similar to that in humans (25). The leading *Leishmania* vaccine candidate (HKLV) has proven safe but poorly immunogenic in clinical trials (26). Coadministration of both D and K ODN with this alum-adjuvanted HKLV vaccine significantly increased the number of PBMC triggered to secrete IFN-γ when stimulated with *Leishmania* Ag in vitro. However, the critical test of any vaccine/adjuvant combination is its ability to induce protective immunity. Results show that the cutaneous lesions of macaques vaccinated with HKLV plus D ODN were significantly reduced when compared with HKLV-alum alone. Previous studies established that a reduction in lesion size correlates with a reduced parasite load (Ref. 32 and R. A. Seder, unpublished observations). These findings suggest that the ability of D ODN to stimulate IFN-α and IFN-γ production while promoting the maturation of APC may be particularly useful for the induction of a protective response against *Leishmania* (17, 18).

K and D ODN have unique structural properties. Optimally active K ODN have a phosphorothioate backbone and express multiple TCGTT and/or TCGTA motifs. D ODN have a mixed phosphodiester/phosphorothioate backbone, express a single self-complementary purine/pyrimidine/CpG/purine/pyrimidine motif, and are capped by a 3′ poly G tail. These two types of ODN trigger human and rhesus PBMC to mount distinct responses. K ODN stimulate B cells to proliferate and secrete IgM, plasmacytoid DC precursors to mature and secrete IL-8, and monocytes to produce IL-6 (Fig. 1 and Refs. 17, 18, 34, and 35). By comparison, D ODN trigger plasmacytoid DC to produce large amounts of IFN-α, and directly or indirectly trigger NK cells to secrete IFN-α, and myeloid DC to mature (Fig. 1, Refs. 17–19, and M. Gursel, unpublished observations). We postulate that D ODN may be superior vaccine adjuvants when a Th1-dependent immune response is required, whereas K ODN may excel at the induction of proinflammatory responses.

It is likely that differences in the recognition, uptake, and/or processing of K and D ODN underlie their distinct functional properties. It was recently established that Toll-like receptor 9 plays a critical role in CpG ODN-mediated activation of human and murine immune cells (35, 36). Using HEK 293 cells transfected with human Toll-like receptor 9, our lab confirmed that the recognition of K-type ODN was mediated by this receptor (37). However, our ongoing studies indicate that these transfected cells do not respond to D ODN, suggesting that a second type of receptor may be involved in D ODN-mediated immune activation.

Clinical trials exploring the utility of CpG ODN as vaccine adjuvants, immunotherapeutic agents, and anti-allergens have been initiated (38). Current results suggest that rhesus macaques may be a useful model for evaluating the safety and activity of these agents in vivo. In this context, neither local nor systemic adverse reactions to K or D CpG ODN were detected in any of the animals studied. Moreover, although K ODN similar to those currently in human clinical trials were found to be active in vivo, our results indicate that D may be superior vaccine adjuvants, improving the humoral response and protective efficacy to certain coadministered vaccines.

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Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide

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Abstract: Synthetic oligodeoxynucleotides (ODN) expressing "CpG motifs" show promise as immune adjuvants, antiallergens, anticancer, and immunoprotective agents. Two structurally distinct classes of CpG ODN have been identified that stimulate human PBMC. This work establishes that both types of ODN bind to and are internalized by the same individual B cells, NK cells, and monocytes. However, the intracellular localization of "D" and "K" ODN differs, as does their functional activity: "K" type ODN trigger monocytes and B cells to proliferate and secrete IL-6 and IgM, whereas "D" type ODN induce NK cells to produce IFN-γ and monocytes to differentiate into $CD83^+/CD86^+$ dendritic cells. In monocytes, these two types of ODN (which differ in backbone composition and CpG motif) cross-inhibit one another's activity. Thus, different types of CpG ODN have distinct and in some cases incompatible effects on the same cells, a finding with important implications for the therapeutic use of these agents. *J. Leukoc. Biol.* 71: 813–820; 2002.

Key Words: CpG DNA · B cells · NK cells · monocytes

INTRODUCTION

Bacterial DNA contains unmethylated "CpG motifs" that strongly activate the mammalian immune system [1–5]. Synthetic oligodeoxynucleotides (ODN) containing such CpG motifs stimulate B cells [2, 6], natural killer (NK) cells [7, 8], and professional antigen-presenting cells (APCs) [9–12] to proliferate and/or secrete a variety of cytokines, chemokines, and immunoglobulins (Ig). Animal studies suggest that CpG ODN may be therapeutically useful as vaccine adjuvants, antiallergens, chemotherapeutic, and immunoprotective agents [13–17].

Previously, we demonstrated that two structurally distinct classes of CpG ODN are capable of activating human peripheral blood mononuclear cells (PBMC), a finding since confirmed by others [8, 18, 19]. "K" type phosphorothioate ODN expressing multiple TCGTT and/or TCGTA motifs stimulate human immune cells to proliferate and secrete interleukin (IL)-6 and IgM (referred to as CpG-B by other groups) [20–23]. "D" type ODN, which contain a phosphodiester purine/pyrimidine/CG/purine/pyrimidine motif, capped at each end by a phosphorothioate poly G tail, stimulate NK cells to produce interferon-γ (IFN-γ; referred to as CpG-A by other groups) [8, 18, 19]. Whether these differences in activity reflect variation in cellular binding, uptake, or signaling by "K" versus "D" ODN and whether these two types of ODN interact synergistically or competitively are unknown.

This work compares the ability of "K" and "D" ODN to activate B cells, NK cells, and monocytes. Results indicate that both types of ODN bind to and enter the same immune cells. However, their intracellular localization differs, as does their functional activity. Moreover, these two types of ODN cross-inhibit one another's ability to activate human monocytes. Thus, ODN, differing in CpG motif and backbone composition, have distinct and in some cases incompatible effects on the same immune cells.

MATERIALS AND METHODS

Oligonucleotides and antibodies

ODN were synthesized at the Center for Biologics Evaluation and Research (Bethesda, MD) core facility. Sequences of the CpG ODN used in this study are 5′-TCGAGCGTTCTC-3′ (K23) and 5′-GgtgcatcgatgcaggggGG-3′ (D35) [8]. The control for "K" ODN was 5′-TCAAGTGTTCTC-3′ and for "D" ODN, 5′-GgtgcatctatgcaggggGG-3′. Bases shown in capital letters are phosphorothioate, and those in lower case are phosphodiester. CpG dinucleotides are underlined. All fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, and cychrome-labeled monoclonal antibodies (mAb) were purchased from Pharmingen (San Jose, CA). All ODNs used in this study contained <0.1 U/mg endotoxin.

Cell cultures

PBMC from normal donors (provided by the NIH Department of Transfusion Medicine, Bethesda, MD) were isolated by Ficoll-Hypaque density-gradient centrifugation [8]. Countercurrent centrifugal elutriation was used to isolate monocytes that were >95% pure. Cells ($0.5–4\times10^6$/ml) were cultured in RPMI 1640 containing 5% fetal calf serum (FCS), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.3 mg/mL L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, and 10^{-5} M 2-mercaptoethanol. Cells were stimulated with ODN for 8–72 h with 1–3 μM ODN depending on the assay.

Analysis of cell proliferation

PBMC were cultured in complete medium plus 3 μM ODN for 72 h. To study B-cell proliferation, cells were loaded with 10 nM carboxy fluorescein succin-

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imidyl ester energy (Molecular Probes, Eugene, OR) as described before [24]. Proliferation of CD11c+ monocytes was monitored by adding 10 μM BrdU (Pharmingen) for the last 18 h of culture. Staining for BrdU was performed as recommended by the manufacturers.

Enzyme-linked immunosorbent assays (ELISAs)

Ninety-six-well microtiter plates (Millipore, Bedford, MA) were coated with anticytokine or anti-IgM Ab and blocked with phosphate-buffered saline (PBS)-5% bovine serum albumin [8]. The plates were incubated for 2 h with culture supernatants from PBMC (5×10^5/ml) that had been stimulated for 8–24 h with ODN as described above. IL-6, IFN-γ, and IgM were detected colorimetrically using biotin-labeled antibodies followed by phosphatase-conjugated avidin and a phosphatase-specific colorimetric substrate [8]. The detection limit of the assays was 6 pg/ml IFN-γ, 20 pg/ml IL-6, and 10 ng/ml IgM. All assays were performed in triplicate.

Staining for cell-surface markers and intracellular cytokine

Cultured cells were washed in cold PBS, fixed, and stained with fluorescent-labeled anti-CD69 (24 h), anti-CD25 (72 h), anti-CD83 (72 h), or anti-CD86 (72 h). To detect intracytoplasmic cytokine, cells incubated with ODN plus 10 μg/ml Brefeldin A for 8 h were washed, fixed, permeabilized (as per the manufacturer's instructions; Caltag, S. San Francisco, CA), and stained with 4 μg/ml PE-conjugated anti-IL-6 or 2 μg/ml PE-conjugated anti-IFN-γ (Pharmingen) plus various FITC and Cy-Chrome-labeled surface markers for 30 min at room temperature. Samples were washed and analyzed (20,000–40,000 events) on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) after gating on live cells with proper electronic compensation. The data were analyzed using CELLQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Analysis of cell-surface binding and internalization of ODN

PBMC (4×10^6/ml) were incubated with biotinylated ODN (1–3 μM) for 10 min at 4°C (binding experiments) or 37°C for 1 h (uptake experiments). To detect internalized ODN, surface-bound ODN were blocked with 100 μg/ml "cold" streptavidin. After washing, these cells were permeabilized, fixed, and stained with PE-conjugated streptavidin (1 μg/ml) plus FITC or Cy-Chrome-conjugated cell-surface markers.

Confocal microscopy

Elutriated monocytes (4×10^6/ml) were incubated with Cy-3 or FITC-labeled "K" and/or "D" ODN at 37°C for 1 h. The cells were washed and mounted using the Prolong antifade kit (Molecular Probes) Subcellular localization of Cy3 and FITC-labeled ODN was determined by confocal microscopy under 1000× magnification (LSM5 PASCAL; Carl Zeiss, Thornwood, NY).

RESULTS

Binding and internalization of CpG ODN

The ability of human PBMC to bind and internalize CpG ODN was examined using biotin-labeled K23 and D35 ODN. Both types of ODN bound rapidly to the surface of virtually all human monocytes at 4°C (**Fig. 1**, upper panel). A significant fraction of B lymphocytes (20–45%) and NK cells (10–20%) also bound these ODN. Simultaneous staining with "K" and "D" ODN showed that the same cells were binding both types of ODN. In contrast, interaction with T cells barely exceeded background levels (Fig. 1).

To monitor internalization, PBMC were incubated with biotin-labeled ODN for 60 min at 37°C. Surface-bound ODN were blocked with excess strepavidin, and internalized ODN were detected by staining fixed, permeabilized cells with FITC-

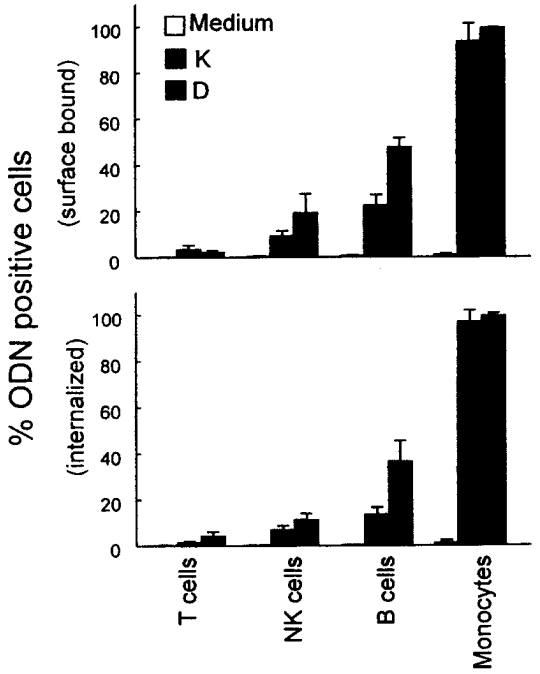

Fig. 1. Binding and uptake of K23 and D35 ODN. Binding studies: Freshly isolated PBMC were incubated with 3 μM biotinylated ODN for 10 min at 4°C, washed, and counter-stained with FITC-avidin and Cy3-labeled phenotype-specific Ab. Uptake studies: PBMC were incubated with 3 μM biotinylated ODN for 60 min at 37°C. Surface-bound ODN was blocked with excess streptavidin, and internalized ODN was detected by FITC-avidin staining of permeabilized cells. Cells incubated with ODN at 4°C showed no internalization of ODN. Data represent the mean percent ± SEM of $CD3^+$ T cells, $CD16^+$ NK cells, $CD19^+$ B cells, and $CD14^+$ monocytes from six independent experiments.

avidin. The fraction of monocytes, B lymphocytes, and NK cells that internalized K23 and D35 ODN was similar to the fraction of each cell type that bound these ODN (Fig. 1). Similar results were obtained using other "D" and "K" ODN (unpublished results). No internalization was observed when cells were incubated with ODN for 10 min at 4°C, suggesting that ODN uptake involves metabolic activity.

The ratio of membrane bound:internalized ODN was compared. Based on differences in mean fluorescence intensity (MFI), we calculate that target cells internalized approximately half of the ODN that had bound to their cell surface (unpublished results). For all cell types, the absolute magnitude of "D" ODN uptake exceeded that of "K" ODN. For example, the amount of labeled "D" ODN that bound to and was taken up by monocytes exceeded that of equimolar "K" ODN by about twofold throughout the functional concentration range of these agents ($P<0.001$; **Fig. 2A**). To achieve equivalent levels of binding and uptake required that "D" ODN be used at a fourfold lower concentration than "K" (e.g., 0.75 vs. 3.0 μM; Fig. 2).

The intracellular localization of these two types of ODN was examined by confocal microscopy of labeled monocytes. As seen in Figure 2B, "K" and "D" ODN largely occupied discrete areas within the same cell, although there was a limited degree of colocalization. "D" ODN largely occupied punctuated vesicles, whereas "K" ODN were distributed more diffusely, stain-

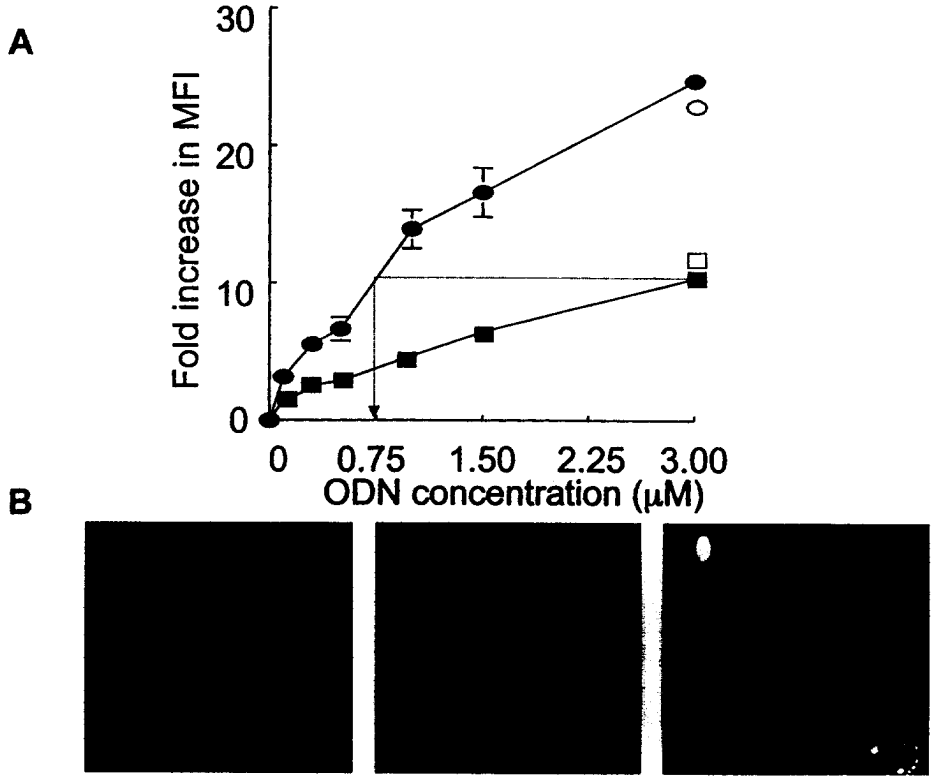

Fig. 2. (A) Internalization and localization of K23 versus D35 ODN. PBMC were incubated with increasing concentrations of biotinylated "K" (■) or "D" ODN (●). A tenfold excess of unlabeled "D" ODN did not inhibit the uptake of labeled "K" ODN (□) nor did unlabeled "K" ODN block the uptake of biotinylated "D" (○). Note that 3 μM "K" and 0.75 μM "D" ODN yield equivalent levels of uptake. Results represent the mean fold increase in MFI over background ± SE from four independent experiments. (B) Subcellular distribution of K23 and D35 ODN in elutriated monocytes. Purified monocytes were incubated with 3 μM Cy-3-labeled "K" (red) and FITC-labeled "D" (green) ODN for 2 h at 37°C. The intracellular localization of these ODN was determined by confocal microscopy. Sections are at 1000× original magnification.

ing the nucleus as well as cytoplasmic vesicles. This difference in localization was associated with the presence or absence of a poly G tail, because control (non-CpG) ODN with a poly G tail showed the same distribution pattern as did "D" ODN (unpublished results). In contrast, the fluorescent dyes used did not influence distribution, because switching dyes had no effect on the ODN localization pattern.

Differential effect of "K" versus "D" ODN on B-cell function

Whole PBMC were treated with optimal concentrations of K23 and D35 ODN. "K" ODN activated $CD19^+$ B cells rapidly, reflected by a significant increase in the expression of the CD69 early activation marker and the CD25 late-activation marker ($P<0.001$; **Table 1** and **Fig. 3**). "K" ODN also triggered a greater-than tenfold increase in B-cell proliferation ($P<0.05$), a greater-than tenfold increase in IgM production ($P<01$), and a fivefold increase in the number of B cells secreting IL-6 ($P<0.001$). The effect of K23 exceeded that of D35 (and of a control for the "K" type ODN of the same structure but lacking the critical CpG motif) by more than tenfold in each of these functional assays. However, "D" ODN were not entirely inactive, because they induced a modest increase in CD25 and CD69 expression by $CD19^+$ B cells (Table 1 and Fig. 3).

Differential effect of "K" versus "D" ODN on NK cells

NK cells were identified by their expression of the CD16 surface marker. "D" ODN stimulated approximately 25% of these cells to increase expression of CD25 and CD69 ($P<0.001$; **Table 2** and **Fig. 4**, upper-right vs. lower-right panels). Consistent with previous studies, "D" ODN also triggered a significant increase in IFN-γ secretion by NK cells ($P<0.05$; Table 2). By comparison, neither "K" ODN nor a non-CpG control for the "D" ODN stimulated IFN-γ production significantly. K23 did induce a modest increase in the number of NK cells expressing CD25 and CD69 ($P<0.05$;

TABLE 1. Effect of "K" and "D" ODN on B Cells

ODN type	% of B cells expressing/producing			Proliferation (stimulation index)	IgM (ng/ml)
	CD25	CD69	IL-6		
K	49.9 ± 7.4	35.3 ± 1.9	8.4 ± 1.7	6.0 ± 0.7	11.6 ± 2.9
D	18.9 ± 3.1	22.3 ± 0.3	1.3 ± 0.3	2.1 ± 0.6	1.5 ± 1.3
K + D	54.1 ± 0.2	38.1 ± 5.8	N.D.	5.1 ± 0.5	11.9 ± 2.9
"K" Control	6.3 ± 1.2	13.3 ± 5.8	1.2 ± 0.3	2.3 ± 0.3	0.7 ± 0.5

PBMC were stimulated with 3 μM K23, D35, or "K" control (non-CpG) ODN for 8–72 h. The percent of $CD19^+$ B cells induced to express CD25 and CD69 and secrete IL-6 was determined by cell-surface and intracytoplasmic staining. Proliferation was monitored by CFSE dilution, and IgM levels in culture supernatants were determined by ELISA. Results represent the mean ± SD of five independent experiments.

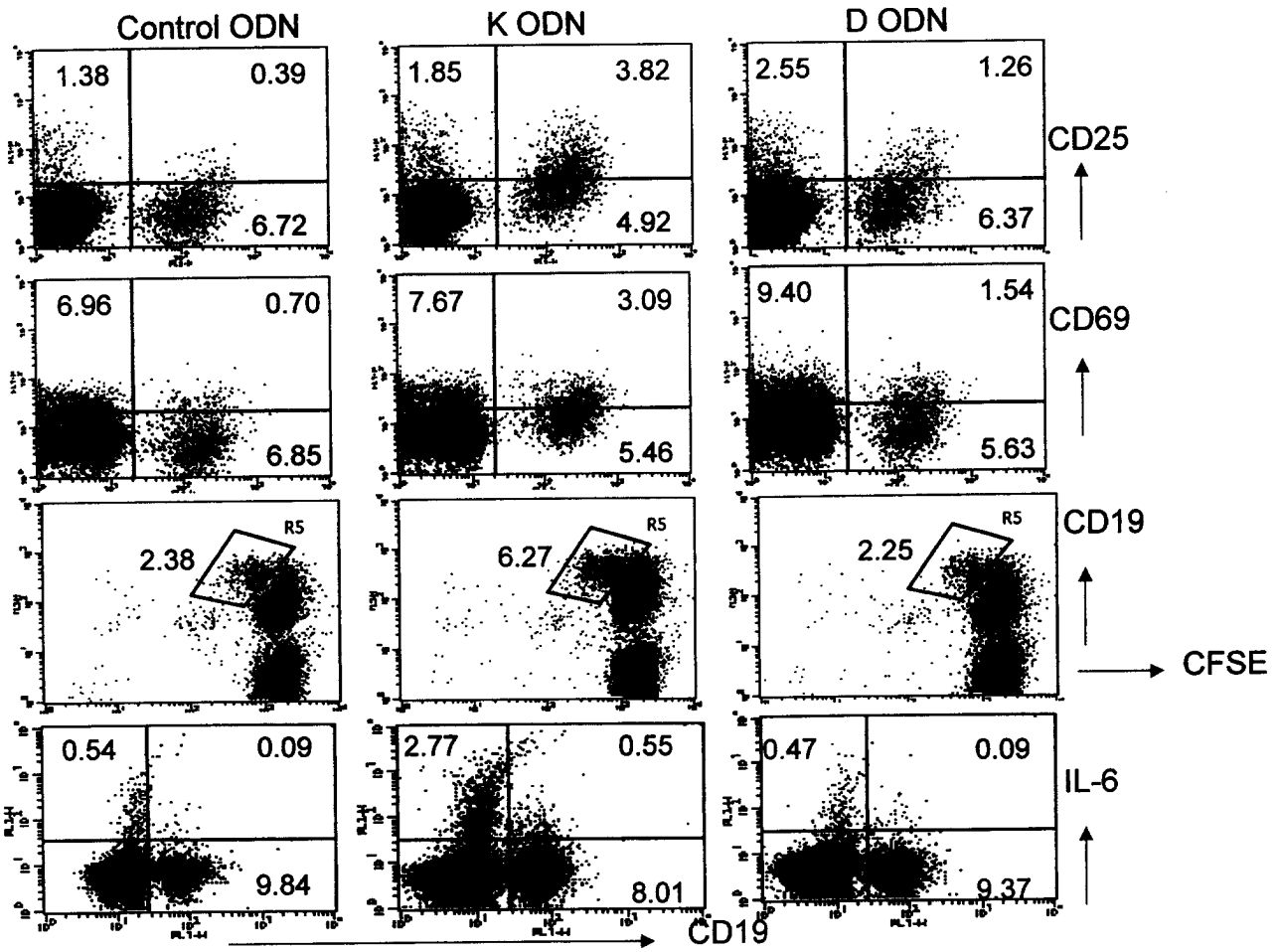

Fig. 3. B-cell activation by K23 versus D35 ODN. PBMC were incubated with 3 μM ODN for 8–72 h. The number of $CD19^+$ B cells induced to up-regulate expression of CD25 and CD69, produce IL-6, and proliferate was monitored by cell-surface and intracytoplasmic staining (see Materials and Methods). Note that in this representative example, the stimulation induced by "K" ODN exceeded that of "D" ODN by every parameter measured.

Table 2). None of these ODN induced NK cells to proliferate (unpublished results).

Differential effect of "K" versus "D" ODN on monocytes

"K" and "D" ODN had disparate effects on purified monocytes. K23 stimulated $CD14^+$ monocytes to proliferate ($P<0.05$) and secrete IL-6 ($P<0.001$), whereas D35 had no effect in these assays (**Table 3** and **Fig. 5**). Instead, "D" (but not "K") ODN stimulated monocytes to mature into $CD83^+/CD86^+$ dendritic cells (DC; $P<0.001$; Table 3 and Fig. 5). The divergent effects of "K" versus "D" ODN on monocytes persisted throughout the physiologic concentration range of both types of ODN and was observed using a variety of "D" and "K" ODN, indicating that these differences were not a result of variation in ODN binding or uptake (unpublished results). Although both types of ODN increased CD69 and CD25 expression, "D" ODN up-regulated these activation markers in monocytes significantly more effectively ($P<0.001$; Table 3).

TABLE 2. Effect of "K" and "D" ODN on NK Cells

ODN type	% of NK Cells Expressing or Producing			IFN-γ (pg/ml)
	CD25	CD69	IFN-γ	
K	15.1 ± 5.9	16.6 ± 1.9	1.8 ± 0.3	1.8 ± 1.2
D	27.8 ± 4.8	25.9 ± 2.1	3.5 ± 0.3	5.5 ± 2.0
K + D	29.3 ± 3.2	25.7 ± 1.6	N.D.	4.3 ± 1.7
"D" Control	3.8 ± 2.4	11.9 ± 0.6	1.6 ± 0.3	0.75 ± 0.5

PBMC were stimulated with 3 μM ODN for 8–72 h. The percent of $CD16^+$ NK cells induced to express CD25 and CD69 or secrete IFN was determined by cell-surface and intracytoplasmic staining. IFN-γ levels in culture supernatants were determined by ELISA. Results represent the mean ± SD of five independent experiments.

Competition between "K" and "D" ODN at the single-cell level

The above findings suggested that monocytes responded differently to stimulation by "K" versus "D" ODN. There are two possible explanations for this observation: These two types of ODN were triggering the same cells to mount distinct types of immune response, or "K" and "D" ODN were acting on different subpopulations of monocytes. The latter explanation seemed unlikely, given that confocal microscopy showed that

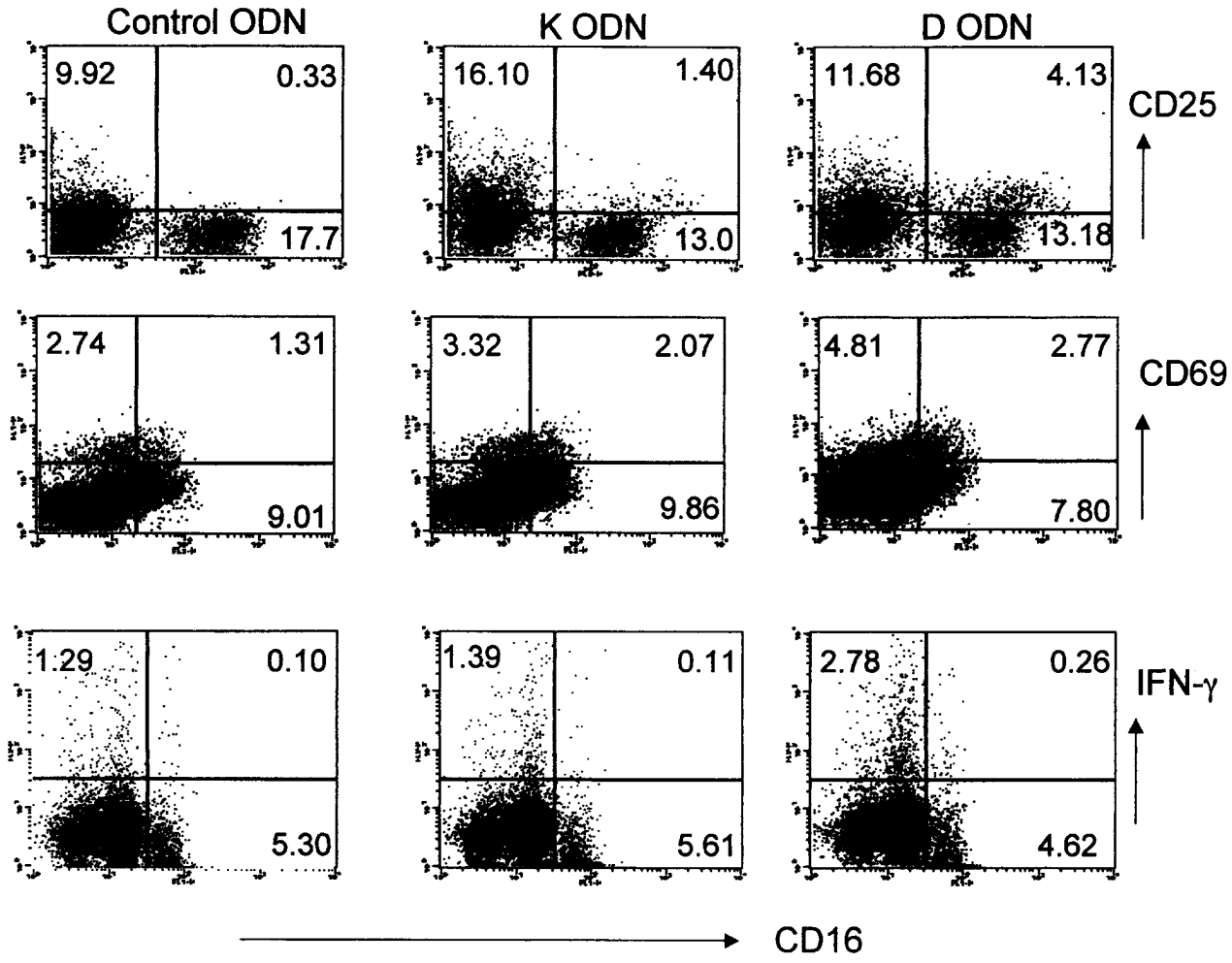

Fig. 4. NK cell activation by K23 versus D35 ODN. PBMC were incubated with 3 μM ODN for 24–72 h. The number of $CD16^+$ NK cells induced to up-regulate expression of CD25 and CD69 and produce IFN was monitored by cell-surface and intracytoplasmic staining. Note that in this representative example, the stimulation induced by "D" ODN exceeded that of "K" ODN in every parameter measured.

the same cells were binding and internalizing both types of ODN (Figs. 1 and 2).

To clarify this situation, monocytes were treated simultaneously with D35 plus K23. At optimally stimulatory concentrations, these ODN did not cross-compete for uptake or binding (Fig. 2A). Yet, when their function was analyzed, coadministration of "K" ODN reduced the ability of "D" ODN to trigger monocyte differentiation by 70% ($P<0.001$; Table 3). The inhibitory effect of "K" ODN on the activity of "D" ODN was sequence-specific and concentration-dependent, because control, non-CpG ODN did not interfere significantly with the activity of "D" ODN (unpublished results). Conversely, "D" ODN significantly reduced the ability of "K" ODN to induce monocytes to proliferate ($P<0.05$; Table 3). As above, the inhibitory effect of "D" on the activity of "K" ODN was sequence-specific and concentration-dependent.

TABLE 3. Effect of "K" and "D" ODN on Monocytes

	% of Monocytes expressing/producing					
ODN type	CD25	CD69	IL-6	IL-6 (ng/ml)	Proliferation (SI)	DC maturation
K	32.8 ± 1.1	39.7 ± 5.3	24.1 ± 2.4	2.0 ± 0.3	7.0 ± 1.4	3.5 ± 0.3
D	48.6 ± 7.5	82.1 ± 2.4	4.6 ± 0.3	0.8 ± 0.1	1.0 ± 0.5	23.7 ± 2.0
K + D	44.7 ± 13.9	53.8 ± 12.5	N.D.	2.2 ± 0.1	2.8 ± 1.7	5.2 ± 0.8
"K" Control	21.8 ± 2.2	30.2 ± 2.5	5.1 ± 0.6	<0.3	2.1 ± 1.6	1.8 ± 0.1

PBMC or elutriated monocytes were stimulated with 3 μM ODN for 8–72 h. The percent of $CD14^+$ monocytes induced to secrete IL-6 was determined by intracytoplasmic staining. Because of down-regulation of CD14, CD11c was used to monitor the expression of CD25 and CD69 by stimulated cells. The percent of $CD83^+/CD86^+$ dendritic cells in culture was determined after 72 h. IL-6 levels in culture supernatants were determined by ELISA, and proliferation was evaluated by BrdU incorporation. Results represent the mean ± SD of five independent experiments.

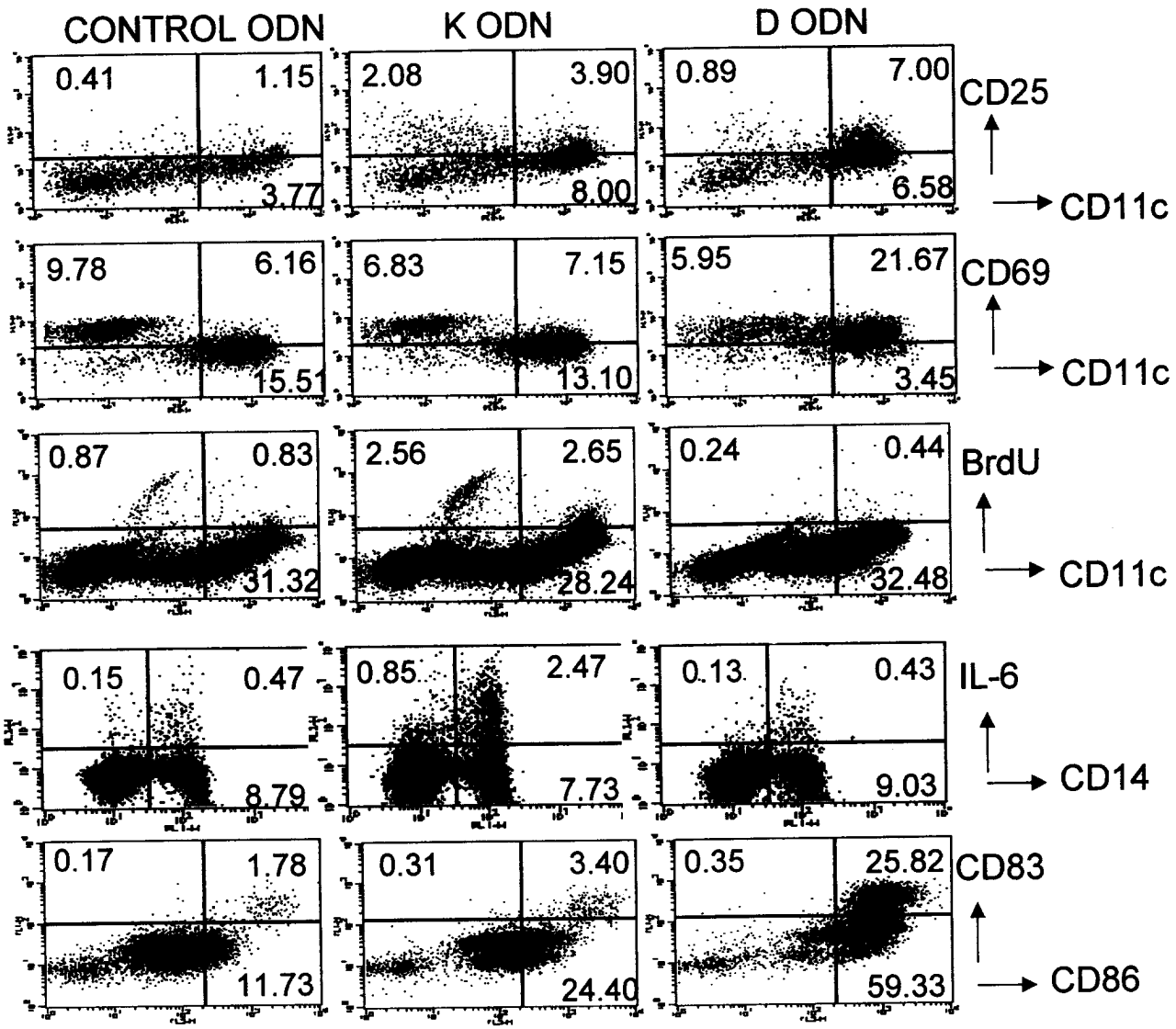

Fig. 5. Monocyte activation by K23 versus D35 ODN. Elutriated monocytes were incubated with 3 μM ODN for 8–72 h. The number of $CD11c^+$ monocytes induced to up-regulate expression of CD25 and CD69 and proliferate was monitored by cell-surface and intracytoplasmic staining. Production of IL-6 by $CD14^+$ monocytes was monitored by intracytoplasmic staining. Note that in this representative example, "K" ODN triggered monocyte proliferation and production of IL-6, and "D" ODN induced monocytes to mature into $CD83^+/CD86^+$ dendritic cells.

A very different pattern emerged when B and NK cells were studied. In these cells, the coadministration of "D" with "K" ODN was not inhibitory. Rather, the ability of "K" ODN to stimulate B cells to proliferate and secrete IL-6 and IgM was unaffected by the presence of "D" ODN, and the ability of "D" ODN to stimulate NK cells to secrete IFN-γ was not reduced by inclusion of "K" ODN.

DISCUSSION

CpG ODN show promise as immune adjuvants and antiallergens and for the treatment of infectious disease and cancer [13–15, 25–29]. Previously, we demonstrated that "K" and "D" ODN might support distinct therapeutic activities. The current work monitored the uptake, distribution, and functional characteristics of "D" versus "K" ODN in multiple cell types. "K" ODN uniquely stimulated B cells to proliferate and secrete, and "D" ODN uniquely activated NK cells to produce IFN-γ. The response of monocytes to these two types of ODN was particularly informative. "K" ODN trigger elutriated monocytes to proliferate and secrete IL-6, and "D" ODN stimulate them to differentiate into dendritic APCs. When mixed, "K" and "D" ODN cross-inhibited one another's effects on monocytes.

The ability of CpG ODN to trigger immune cells to proliferate and secrete Ig and cytokines was documented first in mice [14]. Because of evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents are poorly immunostimulatory in primates [8, 23, 30]. This prompted efforts to identify CpG ODN that stimulated human PBMC, culminating in the discovery of "K" and "D" type ODN. Optimally active "K" ODN are composed of multiple TCGxTT and/or TCGxTA motifs on a phosphorothioate backbone [7, 8, 23, 30–32]. In contrast, "D" ODN consist of a phosphodiester

purine-pyrimidine-CpG-purine-pyrimidine hexamer flanked by self-complementary bases that form a stem-loop structure capped at the 3′ end by a phosphorothioate poly G tail [8]. The ability of "K" ODN to stimulate monocytes and B cells is well-documented, and "K" ODN have entered phase I clinical trials [7, 23, 30–32]. By comparison, much less is known of the functional activity of "D" ODN. The current work not only confirms that "D" ODN induce NK cells to secrete IFN-γ but demonstrates that they also trigger monocytes to mature into $CD83^+/CD86^+$ dendritic cells.

This work is the first to establish that these two different types of CpG ODN can block one another's immunostimulatory activity. We find that "D" ODN were unable to trigger monocytes to differentiate into DC when "K" ODN were present and that the proliferation induced by "K" ODN was disrupted when "D" ODN were present (Table 3). These observations suggest that "D" and "K" ODN trigger competing signaling pathways or that the activation induced by one type of ODN precludes monocytes from responding to the other. Consistent with the latter possibility, "K" ODN stimulate monocytes to proliferate, secrete IL-6, and differentiate into macrophages—effects known to inhibit the maturation of monocytes into DC [33–35].

Of interest, "D" and "K" ODN did not interfere with one another's ability to activate B or NK cells. Both cell types bound and internalized these ODN, yet "K" ODN uniquely triggered B cells to proliferate and secrete, and "D" ODN uniquely stimulated NK cells to produce IFN-γ. Thus, it appears that competition does not occur when "D" and "K" ODN trigger convergent activation pathways.

We and others [36, 37] have demonstrated that "K" ODN interact with Toll-like receptor 9 (TLR-9). Studies show that the cellular activation mediated by "K" ODN involves a signaling cascade in which the serine kinase interleukin-1 receptor-associated kinase interacts with the adaptor protein tumor necrosis receptor-associated factor 6, which in turn links to the mitogen-activated protein-3 kinase transforming growth factor β activated kinase-1. TAK-1 induces the activation of the transcription factor nuclear factor-κB (NF-κB) as well as activated protein-1 transcription family members Jun and Fos, which contribute to the transcription of immune response genes [6, 38, 39]. Unlike "K" ODN, there is no evidence that "D" ODN interact with TLR-9. Rather, ongoing research suggests that the poly G tail of "D" ODN may interact with scavenger receptors on immune cells [40], and studies in our lab indicate that "K" but not "D" ODN stimulate cells transfected with TLR-9 (unpublished results). Consistent with differences in recognition and subsequent uptake, "D" ODN do not compete with "K" ODN for binding or cell entry (Fig. 1). Moreover, "D" and "K" ODN primarily occupy discrete locations within a single cell (although some colocalization is observed; Fig. 2).

K23 and D35 were used as representative "K" and "D" ODN in the current study. However, other "K" and "D" ODN were examined with similar results. As controls, ODN with the same structure as K23 and D35 but lacking the critical CpG motif were used. These controls demonstrate that CpG motifs were responsible for the immune activation observed. Moreover, when the poly G tail of D35 was replaced by a poly T tail, enhanced cellular uptake and immunostimulatory activity were abolished totally, indicating that the poly G component was indispensable for "D" ODN activity. Addition of a poly G tail to K23 altered the uptake and intracellular distribution of this ODN, as a result, decreasing the level of proliferation and IL-6 secretion elicited. Because some lots of FCS (and lipopolysaccharide contamination of any cell-culture reagent) can synergistically enhance the immune activation induced by CpG ODN, all materials used in these studies were prescreened and shown to be immunologically inert in our assays.

"D" and "K" motifs are present in biological products currently undergoing clinical testing (ranging from DNA vaccines to gene-therapy vectors). Preliminary studies suggest that the number and location of CpG motifs can influence the nature and magnitude of the host's immune response to these products. In this context, ongoing studies in our lab suggest that "D" ODN are significantly better than "K" ODN as immune adjuvants in vivo. Knowledge of the specific cell types and functions triggered by these two types of ODN and of their competitive activities should facilitate the rational design of novel and effective therapeutic agents.

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CpG DNA as a vaccine adjuvant

Dennis M Klinman

Synthetic oligodeoxynucleotides containing unmethylated CpG motifs act as immune adjuvants, accelerating and boosting antigen-specific antibody responses by up to 500-fold. CpG motifs promote the production of T-helper 1 and pro-inflammatory cytokines and induce the maturation/activation of professional antigen-presenting cells (including macrophages and dendritic cells). These effects are optimized by maintaining close physical contact between the CpG DNA and the immunogen. Coadministering CpG DNA with a variety of vaccines has improved protective immunity in animal challenge models. Ongoing clinical studies indicate that CpG oligodeoxynucleotides are safe and well-tolerated when administered as adjuvants to humans and in some cases increase vaccine-induced immune responses.

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CONTENTS

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Immunostimulatory properties of CpG DNA

The mammalian immune system is stimulated by exposure to bacterial DNA [1]. This property was first observed in the field of autoimmunity, where animals were stimulated to produce immunoglobulin (Ig)G anti-DNA autoantibodies when exposed to bacterial DNA [2]. More recently, Yamamoto and colleagues demonstrated that fragments of bacterial DNA and synthetic single-stranded oligodeoxynucleotides (ODNs) with sequences patterned after those found in bacteria stimulated the tumoricidal activity of murine natural killer (NK) cells [3,4]. Although initially interpreted as indicating that palindromic sequences in synthetic ODNs activate NK cells [5,6], subsequent studies clarified that the relevant immunostimulatory sequence motif (for mice) consisted of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines [7]. This motif is approximately 20 times more common in bacterial than mammalian DNA, due to differences in the frequency of utilization and methylation pattern of CpG dinucleotides in prokaryotes versus eukaryotes [8,9].

CpG DNA stimulates the immune system of fish, birds and mammals [10]. As will be discussed in greater detail below, the precise hexameric motif that induces maximal immune activation varies between species. In humans, CpG ODNs directly activate B-cells and plasmacytoid dendritic cells (DCs) – and indirectly stimulate additional cell types – to proliferate and/or secrete pro-inflammatory (interleukin [IL]-1, IL-6, IL-18, tumor necrosis factor [TNF]-α) and T-helper (Th)1-associated (interferon [IFN]-γ and IL-12) cytokines [7,11,12]. CpG DNA also induces the polyclonal activation of IgM-secreting cells (including memory B-cells) [7,13,14] and the maturation of professional antigen-presenting cells (APCs) [15]. By comparison, mammalian DNA and ODN lacking unmethylated CpG motifs do not induce cytokine secretion or APC maturation (TABLE 1) [11,15].

Molecular mechanism underlying CpG-induced immune activation

Considerable insight has been gained into the molecular mechanism through which CpG motifs are recognized by and stimulate immune cells. Cells of the immune system express members of the Toll-like family of receptors (TLR). This receptor family mediates responses to

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'pathogen-associated molecular patterns' (PAMPs) expressed by a diverse group of infectious micro-organisms [16]. TLRs consist of an extracellular domain with leucine-rich repeats and an intracytoplasmic domain homologous to the IL-1 receptor [17]. Studies of both murine and human cells indicate that a specific member of the TLR family, TLR9, is responsible for CpG recognition [18,19]. Cells lacking TLR9 are not responsive to CpG DNA and can be made responsive by transfection with TLR9 [19,20].

The interaction between CpG ODN and TLR9 has been monitored using fluorescent probes. When added to the extracellular medium, CpG ODN are rapidly internalized, interacting with TLR9 present in endocytic vesicles [19]. This effect is exquisitely sequence-specific, since eliminating the CpG dinucleotide by inversion or methylation abrogates this interaction [18,19]. The interaction of TLR9 with CpG ODN results in vesicular swelling, acidification and the generation of reactive oxygen species [19,21,22]. This sequence of events is critical to CpG-mediated signaling, since agents that inhibit endosomal maturation or acidification (such as chloroquine and wortmannin) block immune activation [21,22]. Thus, the colocalization of CpG DNA with TLR9 in endosomal vesicles and the accompanying maturation of those vesicles are critical steps in the signaling process.

Cellular activation through members of the TLR family involves a signaling cascade that proceeds through MyD88, IL-1 receptor-activated kinase (IRAK), TNF receptor-associated factor 6 (TRAF6) and culminates in the activation of several transcription factors, including NF-κB, activating protein-1 (AP-1), CCAAT/enhancer binding protein (C/EBP) and cAMP-responsive element-binding protein (CREB), which directly upregulate cytokine/chemokine gene expression [17,23]. MyD88 has been shown to colocalize with TLR9 and CpG ODN [24]. Not surprisingly, dominant negative versions of MyD88, IRAK and TRAF6 inhibit CpG ODN-mediated cellular activation and TLR9 knockout mice fail to mount an immune response when stimulated with CpG ODN [17,23].

Contribution of CpG motifs to the immunogenicity of DNA vaccines

CpG content of DNA vaccines

Vaccine development was invigorated in the 1990s by the finding that mice immunized with antigen-encoding DNA plasmids generated both cellular and humoral immune responses against the encoded antigen. Investigators demonstrated that immunity could be induced against determinants expressed by a variety of viruses, parasites, bacteria and tumors, with protection being generated in some challenge models [25–28].

DNA vaccines consist of an antigen-encoding gene whose expression is regulated by a strong mammalian promoter incorporated into the plasmid backbone of bacterial DNA [25,29,30]. When injected intramuscularly or intradermally, DNA vaccines are transcribed, translated and the protein they encode presented to the immune system in the context of self major histocompatability complex (MHC) [25,29,31].

Although the nature, magnitude and duration of the immune response elicited by DNA vaccines is influenced by multiple factors, intramuscular delivery typically elicits a Th1-driven response, characterized by cytotoxic T-cell (CTL) induction and the release of IFN-γ and antigen-specific IgG2a antibodies [25,32,33]. This pattern is reminiscent of that induced by CpG DNA.

The plasmid vectors that form the backbone of all DNA vaccines are composed of CpG-containing bacterial DNA. Might those CpG motifs contribute to vaccine immunogenicity? This issue was first examined by substituting a CpG-containing amp_r gene for a kan_r selectable marker in a β-galactosidase-encoding plasmid [34]. This re-engineered plasmid elicited a higher IgG antibody response, more CTLs and greater IFN-γ production than the original vector [34,35]. Several laboratories confirmed the finding that adding CpG motifs to the plasmid backbone could improve the immunogenicity of the resultant DNA vaccine (TABLE 2) [35–38].

Effect of CpG motifs on antibody production

The isotype of antigen-specific antibody elicited following plasmid DNA vaccination was compared with that induced by immunization with other immune adjuvants. Whereas protein alone, or protein in complete Freund's adjuvant (CFA), primarily induced the production of IgG1 antibodies (IgG1:IgG2a

Table 1. Immunostimulatory effect of CpG DNA.

	Fold increase in cytokine-secreting cell number			
	IL-6	IL-12	IFN-γ	IgM
E. Coli DNA	3.2 ± 0.2	3.8 ± 0.4	4.7 ± 2.3	3.9 ± 1.1
Calf thymus DNA	0.8 ± 0.2	1.1 ± 0.2	0.8 ± 0.3	0.7 ± 0.2
CpG ODN	5.5 ± 1.1	8.3 ± 1.7	4.7 ± 1.1	4.2 ± 1.6
CpG ODN (Methylated)	0.9 ± 0.2	1.2 ± 0.3	0.8 ± 0.2	1.1 ± 0.2
CpG ODN (DNAse Rx'd)	1.3 ± 0.2	0.8 ± 0.2	1.1 ± 0.2	0.9 ± 0.2
GpC ODN	1.2 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3
Suppressive ODN	1.1 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	1.0 ± 0.1
CpG + suppressive ODN	0.9 ± 0.2	1.0 ± 0.1	1.3 ± 0.3	0.8 ± 0.2

BALB/c spleen cells were incubated with 50 μg/ml of heat denatured *E. coli* DNA, calf thymus DNA, or with 1 μM of stimulatory or control phosphorothioate ODNs. The effect on cytokine production was determined after 10 h by ELISPOT assay [11,33]. Data represent the fold increase in the number of cytokine-secreting cells over background. Results represent the mean ± SD of at least three independent experiments.
E. Coli: Escherichia coli; IFN: Interferon; Ig: Immunoglobulin; IL: Interleukin; ODN: Oligodeoxynucleotides.

Table 2. Effect of CpG motifs on DNA vaccine immunogenicity.

	Fold increase		
	Ab titer	G2a:G1 ratio	IFN-γ
50 µg Plasmid DNA vaccine	39	3.1	5.1
50 µg DNAse treated DNA vaccine	2	ND	1.1
50 µg 80% methylated DNA vaccine	6	ND	1.8
50 µg 97% methylated DNA vaccine	1	ND	0.9
50 µg 97% methylated DNA vaccine + CpG ODN	19	ND	4.8
2 µg plasmid DNA vaccine	3	ND	0.8
2 µg CpG-enriched DNA vaccine	52	ND	3.9
50 µg CpG-enriched DNA vaccine	54	ND	4.1

BALB/c mice were immunized with 2 or 50 mg of plasmid DNA. The effect on serum IgG anti-CS.1 antibody titer and on the number of T-cells/10^6 splenocytes that were activated to secrete IFN-γ *in vitro* by restimulation with antigen, is shown 3 weeks postimmunization. Ab: Antibody; IFN: Interferon; Ig: Immunoglobulin; ND: Not determined; ODN: Oligodeoxynucleotide.

ratio >5), intramuscular injection of DNA vaccines encoding the same proteins preferentially stimulated IgG2a antibody production (IgG1:IgG2a ratio < 1) [37]. Since IFN-γ promotes IgM–IgG2a isotype switching, these findings are consistent with CpG-induced IFN-γ production contributing to the preferential production of IgG2 antibodies in immunized mice (TABLE 2).

Effect of CpG motifs on cytokine production

Mice primed and boosted with DNA vaccines mount an IFN-γ-dominated Th1 immune response [33,34]. When spleen cells from DNA-vaccinated animals were restimulated *in vitro* with immunodominant T-cell epitopes present on the encoded protein, their production of IFN-γ (but not IL-4) significantly increased (TABLE 2). This pattern is consistent with CpG-driven immune activation.

Dose effect of CpG motifs

CpG-enriched plasmids remain immunogenic at doses at which plasmids containing fewer CpG motifs fail to induce an immune response. For example, a DNA vaccine encoding the circumsporozoite protein (CSP) of malaria (termed 1012/PyCSP) was stongly immunogenic at an optimal dose of 50 µg/mouse but lost activity when administered at 2 µg/mouse. When the plasmid backbone was modified by the addition of three to four more CpG motifs, immunogenicity at 2 µg/ml was achieved (TABLE 2) [36].

It is noteworthy that the ability of CpG motifs to augment antibody (Ab) and cytokine production *in vivo* appears to be limited. For example, the immune response induced by a CpG-optimized plasmid is no greater than that of a conventional plasmid when both are administered at high concentration (TABLE 2) [36]. Similarly, dose–response studies indicate that the ability of CpG ODN to stimulate spleen cells to secrete cytokine and Ig *in vitro* reaches a plateau and then begins to fall. In this context, there is evidence that adding too many CpG motifs to the backbone of a plasmid vector may reduce its immunogenicity. In one report, introducing 16 additional CpG motifs into a DNA vaccine improved the humoral immune response elicited *in vivo*, while introducing 50 such motifs reduced the response [39]. This may reflect the ability of CpG motifs to stimulate IFN-γ production – a cytokine that reduces the activity of the CMV promoter used to drive expression of the gene encoded by the DNA vaccine. These findings suggests that the maximal stimulatory effect of CpG motifs may be achieved using relatively low doses of DNA.

Deleting CpG motifs from a plasmid vector reduces DNA vaccine immunogenicity

When the CpG motifs present in a DNA vaccine are methylated with Sss I CpG methylase, their ability to induce cytokine production is significantly reduced (TABLE 2) [33,36,40]. As the duration of Sss I treatment is increased, greater methylation is achieved [36,41]. Increasing the percent methylation of CpG dinucleotides in DNA vaccines progressively reduces their ability to activate cytokine-secreting cells *in vitro*. For example, an 80% methylated vaccine induces 60–80% less cytokine production than does native plasmid, whereas 97% methylated material induces virtually no cytokine production (TABLE 2) [36]. Highly methylated DNA vaccines are also less immunogenic *in vivo*. As seen in TABLE 2, the IgG anti-PyCSP antibody response induced by 97% methylated material *in vivo* was only 3% of control values. These effects can be reversed by coadministering CpG-containing ODN with a CpG-depleted plasmid (TABLE 2).

Immunosuppressive motifs in DNA vaccines

Whereas CpG-containing bacterial DNA causes immunostimulation *in vivo* and *in vitro*, when mammalian DNA is coadministered with CpG DNA, it blocks such activation in a dose-dependent manner (TABLE 1) [38,42]. This suppression may account for the inability of mammalian DNA, which contains a

low frequency of CpG motifs, to induce immune stimulation. Several laboratories showed that mammalian and viral DNA contain motifs that suppress the CpG-induced activation [38,42]. More recent studies indicate that eliminating suppressive motifs from the backbone of a DNA vaccine can improve its immunogenicity by up to threefold [38]. These observations suggest that DNA can both stimulate and suppress the immune system.

CpG ODN act as adjuvants when coadministered with conventional protein antigens

Building upon results implicating CpG motifs in the immunogenicity of DNA vaccines, the efficacy of CpG ODNs as immune adjuvants for conventional proteins and vaccines was examined. Early studies showed that coadministering CpG ODN with model proteins, such as ovalbumin (OVA), hen egg white lysozyme and β-galactosidase, improved serum antibody and IFN-γ titers (TABLES 3 & 4) [35,37,43]. This effect was CpG specific, since control ODN did not boost immunity.

Immunogenicity is optimized by colocalizing antigen with CpG ODN

The first experiments carried out in this field utilized soluble antigens and ODN that could freely diffuse from the site of injection. Reasoning that the adjuvant-like effect of CpG ODNs might be improved if proximity to antigen was maintained, various strategies were employed to sustain the association between ODN and antigen. Results showed that improved immune responses were increased an additional tenfold when CpG ODN were kept in close contact with antigen, either by direct conjugation or by coincorporation into lipid emulsions/vesicles [35,37,44,45]. These results confirm the intuitive expectation that optimal stimulation occurs when antigen and adjuvant are presented to the immune system in close spatial and temporal proximity.

Effect of CpG ODN on antibody production

Consistent with studies of DNA vaccines, CpG ODN altered the isotype of antibody elicited following immunization. For example, OVA alone triggered a primarily IgG1 antibody response, whereas the addition of CpG ODN significantly increased the production of IgG2a antibodies, increasing the IgG2a:IgG1 ratio by ninefold (TABLE 3). A similar shift in isotype profile was observed when a variety of other antigens (including polysaccharide antigens) were coadministered with CpG ODN (TABLE 4) [35,37,44,45].

Effect of CpG ODN on cytokine production

The effect CpG ODN on antigen-specific cytokine-producing cells was also examined. Coadministration of CpG ODNs with OVA increased the number of spleen cells actively secreting IFN-γ *in vivo* by twofold compared with mice immunized with OVA alone (TABLE 3) [37]. To establish that this effect was antigen specific, cells from immunized mice were restimulated *in vitro* with OVA. There was a significant dose-related increase in IFN-γ production by cells from mice immunized with CpG ODN plus antigen (TABLE 3). In contrast, splenocytes from animals immunized with OVA plus control ODN showed no increase in IFN-γ production.

CpG ODN improve vaccine-induced responses

Subsequent studies established that CpG ODN could boost the response elicited by conventional vaccines. This effect was of particular relevance for those pathogens known to be eliminated by a strong Th1 response [46,47]. When CpG ODN were coadministered with vaccines against influenza virus [48], measles virus [49], hepatitis B surface antigen (HbsAg) [50,51] or tetanus toxoid [52], antigen-specific Ab titers rose by up to three orders of magnitude (TABLE 4). CpG ODN also triggered the preferential production of IFN-γ-dependent IgG2a antibodies (Abs) and facilitated the development of antigen-specific CTL [48–51,53,54]. While protection was induced against many of these pathogens in animal challenge models, increased immune activity was not always associated with improved vaccine efficacy. Mice treated with CpG ODN plus influenza vaccine, for example, were not well protected from viral challenge [48].

CpG ODN improve mucosal immune responses

Since many pathogens gain access to the host through the respiratory, gastrointestinal, vaginal, or rectal mucosa, the ability of CpG ODN to boost mucosal immunity was also examined. Administering a combination of CpG ODN plus formalin-inactivated influenza virus intranasally significantly increased flu-specific Ab levels in the serum, saliva and the genital tract [48]. Similarly, intranasal delivery of CpG ODN plus HbsAg or β-galactosidase stimulated strong antigen-specific IgA responses throughout the mucosal immune system and in the serum [55,56]. Spleen cells from intranasally immunized mice preferentially produced IFN-γ rather than IL-4 when re-exposed to

Table 3. CpG ODN increase the immune response to a protein antigen.

		Fold increase	
	Ab titer	G2a:G1 ratio	IFN-γ
OVA alone	18	0.1	2.3
OVA + Cpg ODN	46	0.4	4.7
OVA + GpC ODN	16	0.1	2.1
OVA conjugated to GpC ODN	180	0.9	9.4
(OVA + CpG ODN) emulsified in IFA	140	0.8	8.8

BALB/c mice were immunized and boosted with 20 μg of ovalbumin plus 50 μg of ODN. The ODN were either mixed in the same syringe, conjugated to the OVA via biotin–avidin bridges, or emulsified in IFA. Three weeks after treatment, antigen-specific serum antibody titers were determined by ELISA. At the same time, the number of cells stimulated to secrete IFN-γ/10^6 spenocytes was monitored by ELISPOT assay. Ab: Antibody; IFA: Incomplete Freund's adjuvant; ODN: Oligodeoxynucleotides; OVA: Ovalbumin.

Table 4. CpG ODN as immune adjuvants: use with conventional amtigens

Antigen	Cytokine			
	Fold increase in Ab titer	Ig profile	Profile	Ref.
Ovalbumin	>7-fold (3 weeks)	G2a > G1	IFN-γ	[37,44]
Hen eggwhite lysozyme	>10-fold (3 weeks)	G2a > G1	IFN-γ, IL-5	[43]
Hepatitis B surface antigen	$>10^4$ (4 weeks)			[50]
Influenza virus	10-fold (4 weeks)			[48]
Measles virus	20-fold (4 weeks)	G2a > G1	IFN-γ, IL-5	[49]

This table provides an overview of the type and magnitude of immune response elicited when CpG ODN are coadministered with conventional protein- or vaccine-based antigens. Ab: Antibody; IFN: Interferon; IL: Interleukin.

antigen *in vitro*. They also generated MHC-restricted, antigen (Ag)-specific CTL, replicating the effects of parenterally injected CpG ODN plus Ag [55].

CpG ODN improve the immune response of neonatal animals

The adjuvant-like properties of CpG ODN observed in adult mice triggered interest in their potential to improve the response of newborn animals. Due to the immaturity of the neonatal immune system, newborns exposed to foreign antigens are at risk of mounting an inadequate immune response [57,58], or of developing tolerance rather than immunity [59,60]. For example, newborns respond poorly when immunized with HbsAg, attenuated measles virus, or tetanus toxoid [49,51] and develop tolerance to a DNA vaccine encoding the CSP of malaria [60,61]. Several studies showed that CpG ODN-enhanced Ab and CTL responses when coadministered with antigen to very young mice [49,51]. The interpretation of these findings is complicated, however, because animals in these studies were immunized at multiple ages, obscuring the effect of a single early dose of CpG ODN on subsequent immune responses.

Identification of CpG motifs that are immunostimulatory in primates

Over evolutionary periods, TLR9 molecules expressed by different species have diverged. For example, the TLR9 of mice differs from that of humans by 24% at the amino acid level [18]. Due to this divergence, the precise sequence motif (CpG dinucleotide plus flanking regions) that optimally stimulate immune cells from one species may differ from the optimal sequence in another species. For example, cells transfected with murine TLR9 are more responsive to CpG motifs optimized for activity in mice (GACGTT) than humans (TCGTT)[18–20]. The same cells transfected with human TLR9 responded optimally to TCGTT rather than GACGTT. These findings confirm the importance of TLR9 as a receptor for CpG DNA (described above) and suggest that this receptor may account for species-specific differences in the response to bacterial DNA [19].

Peripheral blood monocytes (PBMC) from humans and other primates (e.g., rhesus macaques, chimpanzees) respond to at least two structurally distinct classes of CpG ODN [62,63]. 'K'-type ODN (also referred to as 'B'-type) have phosphorothioate backbones, encode multiple TCGTT and/or TCGTA motifs, trigger the maturation of plasmacytoid DCs and stimulate the production of IgM and IL-6 (TABLE 5) [17,19,62–64]. D ODN (also referred to as 'A'-type) have mixed phosphodiester/phosphorothioate backbones and contain a single hexameric purine/pyrimidine/CG/purine/pyrimidine motif flanked by self-complementary bases that form a stem–loop structure capped at the 3′ end by a polyG tail [62]. D ODN trigger the maturation of APCs and preferentially induce the secretion of IFN-α and IFN-γ [62,64]. Preliminary studies suggest that additional classes of CpG ODN may exist that can stimulate both B-cells and pDCs.

The response of PBMC from nonhuman and human primates to K- and D-type ODN are quite similar [65–67]. PBMC from both species respond to the same sequence motifs by proliferating and secrete cytokines. In contrast, control ODNs of the same general structure as CpG ODN but lacking the critical CpG dinucleotide do not stimulate PBMC from any primate species.

In vivo *adjuvant activity of CpG ODN in nonhuman primates*

Building on the observation that nonhuman primates respond to the same CpG ODN that stimulate human cells, rhesus macaques were immunized and boosted with a mixture of OVA plus ODN. Animals immunized with antigen and D ODN increased their IgG anti-OVA response by 470-fold after primary ($p < 0.05$) and by 600-fold after secondary ($p < 0.01$) immunization. By comparison, K ODN boosted the IgG Ab response by sevenfold after primary and 35-fold after secondary immunization when compared with pretreatment values ($p < 0.05$). Macaques immunized with OVA plus control ODN generated only a fourfold increase in anti-OVA titer. These findings indicate that 'D' ODN are particularly effective at boosting the antigen-specific humoral response to a coadministered antigen.

Effect of CpG ODN on the immunogenicity & protective efficacy of vaccines in nonhuman primates

Several studies confirm that CpG ODN can act as immune adjuvants in nonhuman primates [63,66,67]. This includes evidence that K-type ODN boost the antigen-specific serum IgG response to alum-adjuvanted hepatitis B vaccine and to a peptide from the

Table 5. Antibody titers of rhesus macaques immunized with OVA plus ODN.

	IgG anti-OVA serum titer	
	Primary	Secondary
OVA + control ODN	460	420
OVA + K-type CpG ODN	780	1250
OVA + D-type CpG ODN	9500	13,800

Macaques were immunized with 4 μg of OVA plus 125 μg of alum and 250 μg of ODN. Values represent the geometric mean titer determined by ELISA. The response of animals immunized with OVA plus alum plus D-type ODN was significantly increased over both other groups ($P < 0.01$).
g: Immunoglobulin; ODN: Oligodeoxynucleotide; OVA: Ovalbumin.

CSP of malaria in orangutans and/or aotus monkeys [63,66,67]. Of particular interest, orangutans (which typically mount poor responses to the haptitis B vaccine alone) generated protective anti-Hepatitis B Ab levels when immunized with the vaccine plus CpG ODN.

Despite evidence of enhanced immunogenicity, early experiments did not establish whether the immune responses generated by adding CpG ODN to a vaccine could boost protection against infection in primates. To evaluate this issue, rhesus macaques were immunized with a leading leishmania vaccine candidate, heat-killed leishmania vaccine (HKLV). Cutaneous leishmania infection provides a useful model for studying protective efficacy, since the nature, severity, duration and histopathology of this infection in macaques is quite similar to that in humans [68,69]. Clinical trials have shown that HKLV alone is safe but poorly immunogenic [69]. Macaques were therefore immunized and boosted with a mixture of HKLV plus CpG ODN. When PBMC from these animals were restimulated *in vitro* with leishmania antigen, those from macaques immunized with HKLV plus CpG ODN had significantly more cells activated to secrete IFN-γ ($p < 0.05$) [65]. In contrast, animals immunized with HKLV alone showed no increased IFN-γ production when compared with unimmunized controls.

The critical measure of an antigen/adjuvant combination is its ability to induce protective immunity. Vaccinated animals were therefore challenged with 10^7 *L. major* metacyclic promastigotes. Animals vaccinated with HKLV alone developed typical cutaneous lesions with a peak surface area of 300 ± 60 mm^2 26 days after challenge (FIGURE 1). Monkeys vaccinated with HKLV plus K ODN developed lesions of similar size, although the peak lesion formation was slightly delayed. Animals immunized with HKLV plus D ODN had significantly smaller lesions (maximal size 80 ± 13 mm^2, $p < 0.05$), consistent with a reduced parasite burden (FIGURE 1) [70]. These findings indicate that CpG ODN can improve the protective efficacy of vaccines in primates as well as rodents.

Safety & activity of CpG ODN in humans

Several reports indicate that CpG ODN may have toxic effects when administered in conjunction with lipopolysaccharides or D-galactosamine [71,72]. Other studies suggest that CpG ODN can promote the development of organ-specific autoimmune disease when coadministered with certain self antigens [73–75]. To examine their toxicity, CpG ODN at a dose equal to exceeding that typically used in adjuvant experiments were injected weekly for 4 months into normal BALB/c mice. All of the animals remained physically vigorous [76]. None of the animals became sick, lost weight, or died. Cohorts of mice sacrificed 1–30 days after the end of treatment were examined histologically. None showed macroscopic or microscopic evidence of tissue damage or inflammation [76]. Similarly, no adverse health effects have been reported in studies involving the delivery of CpG ODN to nonhuman primates [65].

Two clinical trials involving the use of K/B-type CpG ODN as vaccine adjuvants for vaccines against infectious diseases have been conducted. In the first double-blind study, 0, 0.5 or 1 mg of CpG ODN was coadministered with the licensed hepatitis B vaccine (Engeirix-B™, GlaxoSmithKline [GSK], MD, USA) intramuscularly to healthy, nonimmune adult volunteers on weeks 0, 4 and 24. Addition of CpG ODN resulted in significantly earlier and stronger serum anti-HBs antibody responses when compared to volunteers immunized with vaccine alone. Two weeks after initial immunization, anti-HBs titers could be detected in >80% of subjects receiving vaccine plus CpG ODN but in none of the control group. The geometric mean titer of anti-HBs Abs among subjects treated with CpG ODN plus vaccine were 13–45-fold higher than in recipients of vaccine alone after both primary and secondary immunization.

In the second double-blind study, 1 mg of CpG ODN was coadministered intramuscularly with either the standard or one tenth the standard dose of the Fluarix™ (GSK, MD, USA) influenza vaccine. When compared with subjects receiving the same dose of Fluarix alone, the addition of CpG ODN had no effect on the response of naive recipients but significantly increased the anti-HI titers among subjects with pre-existing antibody levels. PBMC from CpG ODN vaccinated subjects responded to *in vitro* restimulation by secreting significantly higher levels of IFN-γ than PBMC from control vacinees.

Adverse events (AEs) were observed in all groups of vaccine recipients. These consisted predominantly of injection site reactions (such as pain and erythema) and flu-like symptoms. These symptoms were short lived and did not interfere with the activities of daily living. The intensity of the AEs was similar in all groups, although the frequency of AEs was higher among those vaccinated with CpG ODN plus vaccine (versus vaccine alone). No serious AEs attributed to use of CpG ODN were reported. There were no clinically relevant changes in hematocrit or white blood cell count among immunized volunteers, nor were there any changes in liver or renal function. Despite evidence from animal studies that polyclonal B-cell activation and the production of Th1 cytokines stimulated by CpG ODN may predispose to the development of systemic or organ-specific autoimmune disease, none of the subjects exposed to CpG ODN in these trials developed signs or symptoms of autoimmune disease.

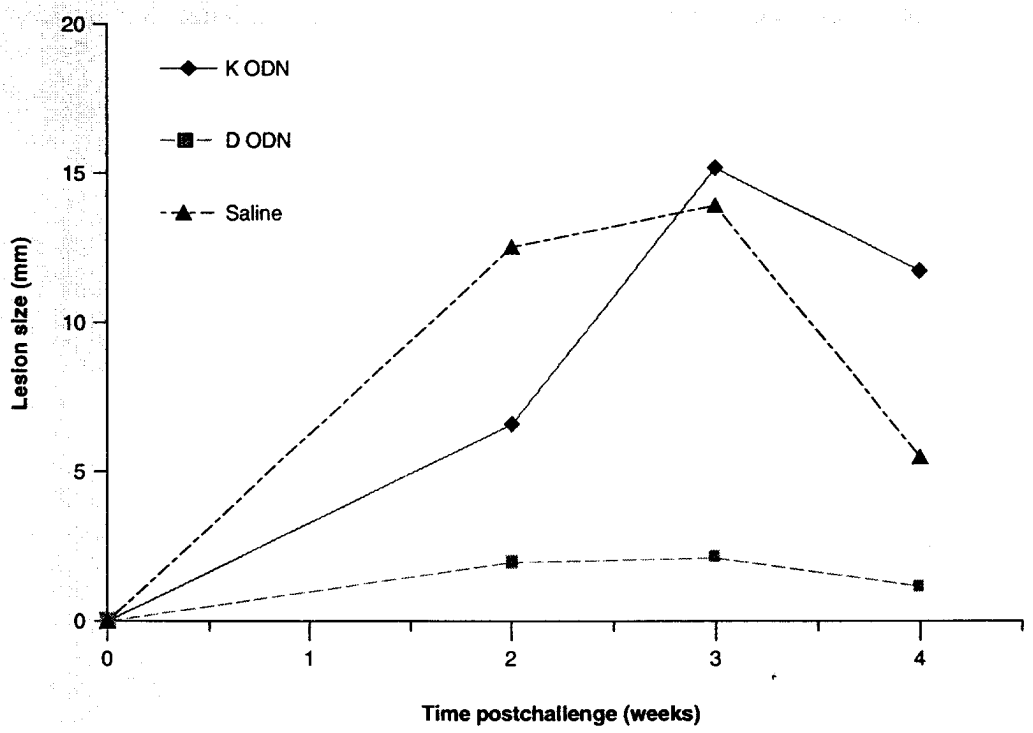

Figure 1. Cutaneous lesions in macaques vaccinated with alum-adjuvanted HKLV plus ODN. Rhesus macaques (n = 6/group) were primed subcutaneously and boosted 4 weeks later with 250 μg of alum-adjuvanted HKLV alone or combined with 500 μg of a mixture of 'D' type or 'K' type CpG ODN. Animals were challenged with 10^7 metacyclic promastigotes on week 14. The average size of the lesions on the forehead (the site of challenge) is shown as the mean area (calculated as [mean diameter/2]^2 × π). Note that macaques immunized with HKLV plus 'D'ODN had significantly smaller lesions ($p<0.01$). PBMC from animals immunized with HKLV plus CpG ODN also produced significantly more IFN-γ than animals immunized with HKLV alone ($p < 0.05$, data not shown).
HKLV: Heat-killed leishmania vaccine; ODN: Oligodeoxynucleotide; PBMC: Peripheral blood monocyte.

Expert opinion & five-year view

An important goal of vaccine research is to identify agents capable of boosting antigen-specific immunity. CpG ODN show promise as vaccine adjuvants, based on their ability to stimulate an innate immune response characterized by the proliferation/activation of professional APC and B-cells and the production of pro-inflammatory (IL-1, IL-6, IL-18, TNF-α) and Th1-associated (IFN-γ and IL-12) cytokines.

The immune system of species ranging from fish to primates recognize and respond to CpG ODN. ODN enter immune cells within seconds, upregulate mRNA within minutes and stimulate the release of immunoprotective cytokines and polyreactive IgM Abs within hours of administration (FIGURE 1) [6,11,77,78]. Exposure to CpG DNA triggers an immunomodulatory cascade that culminates in the stimulation, directly or indirectly, of multiple types of immune cell. They improve the antigen presenting function of DCs, monocytes and macrophages, induce the proliferation of B-cells, boost Ab and cytokine production by antigen-activated lymphocytes, stimulate the immunoprotective activity of NK cells and recruit T-cells to the site of administration [44,79–81]. The capacity of CpG ODN to modulate the host's cytokine milieu contributes to their activity as adjuvants. The induction of IL-6 promotes B-cell activation, IFN-γ and IL-12 support the generation of Th1-dependent immune responses and CTL, while IL-18 and TNF-α improve APC function.

Clinical and preclinical studies typically utilize phosphorothioate-modified ODN as immune adjuvants, since they have superior stability characteristics, reduced susceptibility to DNAse digestion and a longer *in vivo* half-life. Phosphorothioate ODN are readily manufactured at high purity and low cost, are stable at room temperature and are easily reconstituted for rapid administration. They are also intrinsically immunostimulatory, which further boosts the cytokine and IgM production elicited by the CpG motifs they express [82–84].

Available evidence supports the conclusion that CpG motifs present in DNA vaccines serve an immunostimulatory function, activating the innate immune system and thereby promoting humoral and/or cell-mediated responses against environmental and plasmid-encoded antigens. These findings are consistent with the model proposed by Fearon and Locksley, who postulated that an innate immune response can create an immune milieu conducive to the development of antigen-specific immunity [85]. Further evidence suggests that CpG DNA can function as an immune adjuvant when coadministered with conventional protein-based vaccines. Preclinical studies involving nonhuman primates confirm the expectation that CpG ODN selected for their ability to activate human immune

cells can boost the immune response to coadministered vaccines. While the magnitude of this effect varies with the type of antigen and ODN utilized, studies with heat-killed leishmania vaccine indicate that CpG ODN can convert an ineffective vaccine to one that provides significant protection from infection [65]. Clinical studies designed to evaluate the safety and activity of CpG ODN in humans are ongoing. Available results suggest that these agents are safe and can in some cases boost immunity to coadministered vaccines.

The utility of CpG ODN is underscored by their ability to enhance mucosal as well as systemic immunity. This is of considerable importance when dealing with pathogens, such as respiratory syncytical virus, that gain access to the host through the respiratory tract. Several studies show that combining CpG ODN with vaccines significantly increases antigen-specific IgA levels at mucosal sites (including the respiratory, GI and reproductive tracts) and IgG levels systemically [48,50,55]. An additional benefit of CpG ODN is their ability to boost immunity in groups with reduced immune function, such as newborns, the elderly and the immunosuppressed. For example, studies indicate that CpG ODN administered to young animals can boost serum Ab levels and/or improve Th1 responses to coadministered vaccines [49,51]. By activating professional APCs and stimulating the production of Th1 and pro-inflammatory cytokines, CpG ODN apparently create an immune milieu that facilitates the induction of antigen specific immunity.

Efforts are underway to:

- Identify ODNs of different classes that are optimally active in humans when coadministered with different vaccines
- Determine how these different classes of ODN regulate discrete elements of the immune response [44,86,87]
- Monitor the long-term safety of CpG ODN
- Establish the optimal dose, duration and site(s) of vaccine/ODN delivery

We expect these efforts to further improve the efficacy of CpG DNA in boosting immunity against previously resistant pathogens, including those that may be used in biowarfare [88].

Key issues

- CpG oligodeoxynucleotides (ODN) interact with Toll-like receptor 9 to trigger the maturation and functional activation of professional antigen presenting cells, B-cells and natural killer cells.
- The resultant immune response is characterized by the production of polyreactive immunoglobulin (Ig)M antibodies, cytokines and chemokines, skewed towards the induction of T-helper 1 immunity.
- The presence of CpG motifs in their plasmid backbone contributes to the immunogenicity of DNA vaccines. Optimizing the sequence, number and location of such motifs may improve DNA vaccine efficacy.
- Combining CpG ODN with conventional protein antigens and vaccines leads to significantly higher antigen-specific IgG2a and interferon-γ responses.
- CpG ODN can be used to boost immunity in neonatal, adult and geriatric populations. They are effective vaccine adjuvants when administered either systemically or mucosally.
- CpG motifs optimized for activity in humans have been identified.
- Clinical trials indicate CpG ODN can significantly boost the immunogenicity of coadministered vaccines. Local reactogenicity may increase, but general safety is good.

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CUTTING EDGE

Cutting Edge: Role of Toll-Like Receptor 9 in CpG DNA-Induced Activation of Human Cells[1]

Fumihiko Takeshita,[2,3] Cynthia A. Leifer,[2] Ihsan Gursel, Ken J. Ishii, Saoko Takeshita, Mayda Gursel, and Dennis M. Klinman[4]

Unmethylated CpG motifs present in bacterial DNA stimulate a rapid and robust innate immune response. Human cell lines and PBMC that recognize CpG DNA express membrane-bound human Toll-like receptor 9 (hTLR9). Cells that are not responsive to CpG DNA become responsive when transfected with hTLR9. Expression of hTLR9 dramatically increases uptake of CpG (but not control) DNA into endocytic vesicles. Upon cell stimulation, hTLR9 and CpG DNA are found in the same endocytic vesicles. Cells expressing hTLR9 are stimulated by CpG motifs that are active in primates but not rodents, suggesting that evolutionary divergence between TLR9 molecules underlies species-specific differences in the recognition of bacterial DNA. These findings indicate that hTLR9 plays a critical role in the CpG DNA-mediated activation of human cells. ***The Journal of Immunology,*** **2001, 167: 3555–3558.**

Members of the Toll-like receptor (TLR)[5] family respond to pathogen-associated molecular patterns expressed by a diverse group of infectious microorganisms (1), thereby triggering the host's innate immune system. TLRs contain an extracellular domain with leucine-rich repeats and an intracytoplasmic domain homologous to the IL-1R (2). Cellular activation by TLR proceeds through a signaling cascade involving myeloid differentiation marker 88, IL-1R-associated kinase (IRAK), TNFR-associated factor 6 (TRAF6), and NF-κB translocation, culminating in the up-regulation of genes involved in host defense (2).

Unmethylated CpG motifs are present at a 20-fold higher frequency in bacterial than mammalian DNA, due to a combination of CpG suppression and CpG methylation (3, 4). CpG motifs trigger an innate immune response characterized by the activation of Ig-, cytokine-, and chemokine-secreting cells (3, 5). This response confers protection against a variety of intracellular pathogens consistent with unmethylated CpG motifs acting as pathogen-associated molecular patterns. Preclinical and clinical studies indicate that synthetic oligodeoxynucleotides (ODN) containing CpG motifs may have therapeutic value as immune adjuvants and anti-infectious agents (4, 6).

Recent studies indicate that TLR9 plays a critical role in the recognition of CpG motifs in mice. Dominant-negative (DN) versions of myeloid differentiation marker 88, IRAK, and TRAF6 inhibit CpG ODN-mediated cellular activation, and TLR9-knockout mice fail to mount an immune response when stimulated with CpG ODN (7, 8). Despite 76% identity at the amino acid level between murine and human TLR9 (hTLR9) (7), the CpG motifs that are most active in mice have little effect on human cells, and vice versa (9). Moreover, the type of CpG motif expressed by various pathogens influences the type and magnitude of immune response elicited in different mammalian species (10). The present work examines whether CpG recognition in humans is also mediated by TLR9 and explores the nature of the receptor-ligand interaction.

Materials and Methods

Reagents

Phosphorothioate ODNs were synthesized at the Center for Biologics Evaluation and Research core facility (Bethesda, MD). Sequences of the ODN (5′→3′) were: K3 CpG ODN, ATCGACTCTCGAGCGTTCTC; K3-flip control ODN, ATGCACTCTGCAGGCTTCTC; K3-methyl, AT^{m}CGAC TCT^{m}CGAG^{m}CGTTCTC; 2006, TCGTCGTTTTGTCGTTTTGCTGTT (11); 1466, TCAACGTTGA; 1555, GCTAGACGTTAGCGT; and 1612, GCTAGATGTTAGCGT, where ^{m}C indicates a methyl cytosine. Cy3 was conjugated to the 5′ end of some ODN. LPS was purchased from Sigma (St. Louis, MO). Human IFN-γ was purchased from Life Technologies (Gaithersburg, MD).

Cells and cell cultures

Cell lines (obtained from American Type Culture Collection, Manassas, VA) were maintained in complete DMEM (10% FCS, 50 mg/ml penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES buffer, 0.11 mg/ml sodium pyruvate, and 0.5 mM 2-ME). Elutriated monocytes and PBMC were obtained from the National Institutes of Health Blood Bank (Bethesda, MD).

Plasmid construction

Human TLR9 cDNA (the gift of Dr. B. Beutler, Scripps Research Institute, La Jolla, CA) (12) was inserted into pCIneo (Promega, Madison, WI).

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[5] Abbreviations used in this paper: TLR, Toll-like receptor; IRAK, IL-1R-associated kinase; TRAF6, TNFR-associated factor 6; ODN, oligodeoxynucleotides; hTLR9, human TLR9; DN, dominant negative; HA, hemagglutinin; ICD, intracellular domain; ECD, extracellular domain.

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Human TLR9B (amino acids 58–1032), hTLR9 α5 deletion mutant (1–1000), and hTLR9 intracellular domain (ICD) deletion mutant (1–860) were PCR generated from this cDNA. TLR9 (26–1032) was cloned into pDisplay (Invitrogen, Carlsbad, CA), which generated a hemagglutinin (HA) tag. PCR-amplified DN IRAK1 (1–96) and DN TRAF6 (287–523) were cloned into pFlagCMV4 (Sigma).

Cell transfection and luciferase assay

Cells (5×10^4) were transfected using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) plus 0.1 μg p5xNF-κB-luc (Stratagene, La Jolla, CA), 0.1 μg pSV-β-galactosidase (Promega), and 0.2–0.8 μg of various expression vectors for 18 h. Luciferase assays were performed as recommended by the manufacturer (Promega) after 24 h. β-Galactosidase activity was used to normalize the data.

RT-PCR

PCR (33–40 cycles)-amplified products from 1 to 5 μg of reverse-transcribed RNA were visualized by ethidium bromide staining on agarose gels.

Confocal microscopy

Transfected 293T cells were treated with Cy3-labeled ODN for 10–120 min at 37°C. Cells were washed, fixed, permeabilized, and stained for HA-TLR9 protein using FITC-anti-HA Ab (clone 3F10; Roche Molecular Biochemicals). Subcellular localization of Cy3 and FITC signals were determined by confocal microscopy (LSM5 PASCAL; Carl Zeiss, Thornwood, NY).

Results and Discussion

CpG responsiveness of human cells correlates with TLR9 mRNA expression

TLR9 mRNA was expressed in CpG-responsive human monocytes and RPMI 8226 cells, but not by unresponsive cells (such as Jurkat or 293; Fig. 1). Human PBMC constitutively expressed low levels of TLR9 mRNA. IFN-γ treatment significantly increased both TLR9 mRNA expression and CpG DNA responsiveness of PBMC (Fig. 1 and data not shown). Therefore, TLR9 expression is a prerequisite for cell activation by CpG DNA, and factors that increase TLR9 mRNA levels also increase responsiveness to CpG DNA.

TLR9 confers responsiveness to CpG ODN that activate human cells

To examine the importance of TLR9 in the CpG-mediated activation of human cells, 293 cells were transiently cotransfected with a NF-κB-dependent luciferase reporter (p5xNF-κB-luc) plus hTLR9 (hereafter referred to as 293^{Trans}). 293^{Trans} stimulated with CpG ODN that activate human PBMC (9, 10) significantly increased NF-κB dependent luciferase activity (Fig. 2*A*). This stimulation was abrogated if the critical CpG motif was disrupted by inversion or methylation (Fig. 2*A*). Moreover, CpG ODN known to trigger rodent but not primate cells (ODN 1555 and 1466) were uniformly inactive (Fig. 2*A*). The response of TLR9 was CpG specific, because 293^{Trans} did not respond to LPS (Fig. 2*A*), and 293 cells transfected with TLR4 did not respond to CpG ODN (data not shown). These findings indicate that 1) TLR9 is involved in the recognition of human CpG motifs, and 2) differences in CpG recognition between species may reflect evolutionary divergence between TLR9 molecules.

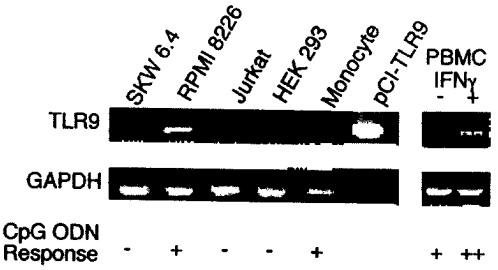

FIGURE 1. TLR9 mRNA expression correlates with CpG ODN responsiveness. RT-PCR specific for TLR9 or GAPDH (*left panel*) was performed on RNA isolated from various human cell lines, elutriated primary monocytes, and the pCIneo-TLR9 plasmid (no reverse transcription). Fresh human PBMC also expressed TLR9 mRNA (*right panel*). When stimulated with 1000 U/ml IFN-γ for 18 h, the responsiveness of these PBMC and their levels of TLR9 mRNA both increased.

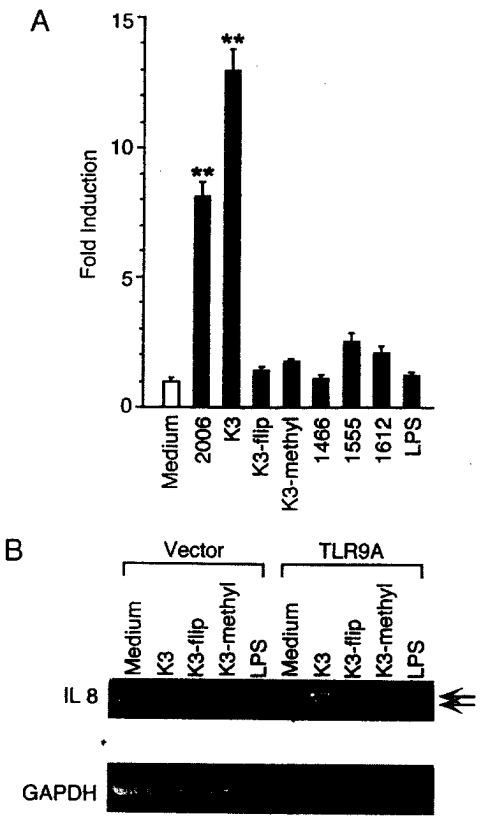

FIGURE 2. Expression of hTLR9 confers CpG ODN responsiveness. *A*, Luciferase activity of transfected cells 24 h after treatment with 1 μM of the following ODN: human-stimulatory CpG ODN (2006 and K3), ODN in which the critical CpG dinucleotide was inverted or methylated (K3-flip and K3-methyl), murine-stimulatory CpG ODN (1466 and 1555), control ODN (1612), or 1 μg/ml LPS. Results represent the mean + SEM of three to five independent experiments; *, $p \leq .01$, and **, $p \leq .001$ compared with identically treated cells cultured in medium. *B*, IL-8 mRNA expression by 293 cells transfected with vector or TLR9 and stimulated for 24 h with 1 μM CpG or control ODN as detected by PCR.

The 293 cells transfected with TLR9 alone responded to CpG ODN by significantly increasing endogenous IL-8 mRNA expression (Fig. 2*B*). Control ODN had no effect on TLR9-transfected cells, and CpG ODN had no effect on mock-transfected cells.

IRAK1 and TRAF6 participate in the TLR9-dependent signaling cascade

DN forms of IRAK1 or TRAF6 inhibited TLR9-mediated luciferase activity in a dose-dependent manner (Fig. 3*A*). Although previous studies established that IRAK1 and TRAF6 were critical for CpG ODN-mediated cell signaling (8), current findings establish that their activation proceeds through TLR9 engagement. In contrast, cofactors known to stabilize cell membrane expression of other members of the TLR family, such as MD1 and MD2 (13), did not influence CpG ODN-mediated activation of 293^{Trans} (data not shown).

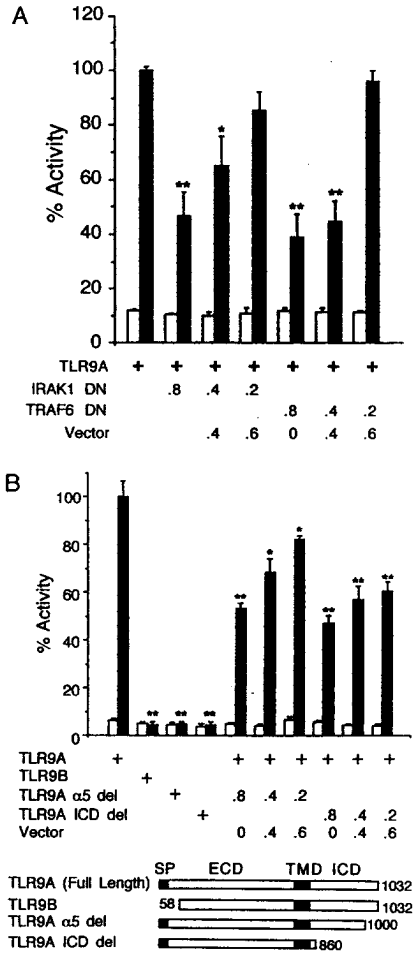

FIGURE 3. CpG ODN-mediated TLR9 signaling. *A*, The 293 cells were cotransfected with p5xNF-κB-luc, TLR9, and varying amounts of DN IRAK1 or TRAF6 plus vector to yield a constant 0.8 μg DNA/reaction. Data show luciferase activity 24 h after incubation in medium (□) or 1 μM CpG ODN (■). *B*, Same as in *A* except 293 cells were cotransfected with p5xNF-κB-luc, TLR9/B, and/or 0.2–0.8 μg of ICD-deleted TLR9 mutants (α5 del or ICD del). The diagram shows TLR9 and truncation mutants. SP, signal peptide; TMD, transmembrane domain. Results represent the mean + SEM of three independent experiments; *, $p \leq 0.05$, and **, $p \leq 0.01$ compared with cells transfected with TLR9 alone.

Contribution of intracellular and extracellular domains to cell signaling

To identify those regions of the TLR9 molecule critical to cell signaling, deletion mutants were generated (Fig. 3*B*). Cells transfected with TLR9B (lacking the NH_2-portion of TLR9; Ref. 12) did not respond to CpG ODN (Fig. 3*B*). As expected, eliminating the entire ICD also abrogated CpG ODN-mediated NF-κB activation (Fig. 3*B*). Interestingly, a TLR9 construct lacking only the C-terminal 32 amino acids of the ICD was also inactive, suggesting that this region plays a critical role in cell signaling.

Because eliminating the extracellular domain (ECD) can constitutively activate TLRs (14), 293 cells were cotransfected with TLR9 plus an ICD deletion mutant to examine the contribution of the ECD to TLR9-mediated signaling/activation. CpG ODN-dependent cellular activation was suppressed in a dose-dependent fashion by both the α5 and ICD deletion mutants (Fig. 3*B*). This effect was most likely mediated by ECD interactions, because cotransfection did not alter TLR9 mRNA expression (data not shown). Thus, similar to other members of the TLR family, TLR9 signaling appears to involve the generation of multimers through ECD interactions (15, 16).

Cellular localization of CpG ODN and TLR9

Several members of the TLR family are expressed on the plasma membrane (13, 17). Signaling through TLR2 involves the redistribution of the receptor from the membrane into phagosomal vesicles (17). Although uptake by acidified endocytic vesicles may be required for CpG-mediated signaling (18, 19), recent reports suggest that CpG ODN can bind to the plasma membrane and need not be internalized to trigger (20).

To examine the relationship between CpG binding, endocytosis, and signaling, a TLR9 construct encoding a HA tag (HA-TLR9) was generated. Cells transfected with HA-TLR9 specifically bound FITC-anti-HA Ab (Fig. 4*A*) and activated NF-κB in response to CpG but not control ODN (data not shown). Cell surface staining of HA-TLR9 transfectants showed that a fraction of the TLR9 is on the cell surface (data not shown). Because transfected 293T cells over-express HA-TLR9, the location of this molecule under physiologic conditions requires further study.

Cells expressing HA-TLR9 were incubated with Cy3-labeled ODN. CpG ODN initially associated with the cell surface, began to form vesicles near the surface, and entered the nucleus of HA-TLR9-transfected cells within 10 min (data not shown). By 2 h, the size and number of CpG ODN-containing vesicles had increased, and the vesicles relocated from near the plasma membrane to intracellular regions (Fig. 4*A*). In some cases, both hTLR9 and CpG ODN were colocalized within the same endocytic vesicle (Fig. 4*A*).

Control (non-CpG) ODN also rapidly gained access to the nucleus and formed small vesicles near the surface of HA-TLR9-transfected cells. However, these vesicles did not change in size or number over time, nor did they relocate within the cell (Fig. 4*B*). Similarly, ODN reached the nucleus of cells transfected with vector alone, but induced minimal vesicle formation (Fig. 4, *C* and *D*). Thus, cells that lack TLR9 can internalize DNA in a sequence-nonspecific manner, but TLR9 enhances vesicular uptake, vesicle relocation, and cellular activation in the presence of CpG motifs.

To verify these conclusions, 293T cells were transiently transfected with HA-TLR9 ICD deletion mutant, a mutant TLR9 lacking the cytoplasmic tail. This mutant does not signal, instead acting

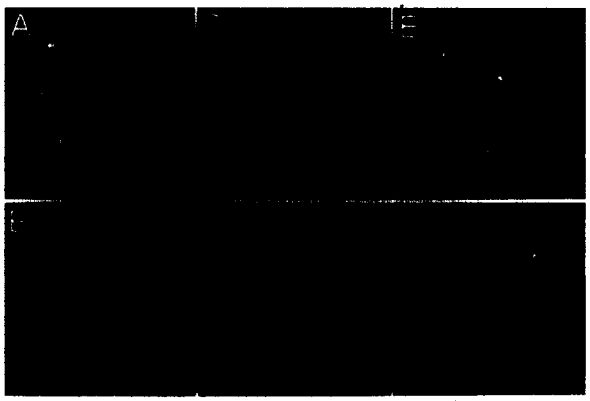

FIGURE 4. Cellular localization of TLR9 and CpG ODN. The 293T cells were transfected with HA-TLR9 (*A* and *B*), vector (*C* and *D*), or HA-TLR9 ICD deletion mutant (*E* and *F*) and treated for 2 h with Cy3-CpG ODN (red; *A*, *C*, and *E*) or Cy3-control ODN (*B*, *D*, and *F*). Cells were then fixed, stained with FITC-conjugated anti-HA Ab (green), and analyzed by confocal microscopy. Arrows show larger yellow/orange vesicles, indicating colocalization; and arrowheads show smaller, more peripheral red vesicles. Representative sections from one of six independent experiments (magnification, ×1000), are shown.

as a DN when coexpressed with hTLR9 (Fig. 3). Similar to vector-transfected cells, ODN gained access to the nucleus but formed only small peripheral vesicles in cells transfected with the HA-TLR9 ICD deletion mutant (Fig. 4, *E* and *F*). Prolonged incubation did not increase in the number or size of CpG-containing vesicles and rarely triggered their relocation. Thus, cellular activation through TLR9 was linked to enhanced vesicular uptake of CpG ODN.

Conclusions

This work provides three fundamental insights into the role of hTLR9 in CpG-mediated activation of human cells. First, expression of TLR9 is a prerequisite for CpG ODN responsiveness. This supports and extends observations in mice that TLR9 plays a critical role in CpG recognition (7). Our results demonstrate that hTLR9 is a cell surface receptor expressed by CpG-responsive cells, and that hTLR9 transfection confers CpG reactivity to cells that are otherwise nonresponsive. Second, hTLR9 enhances vesicular uptake of CpG but not control ODN. In some cases, TLR9 and CpG ODN colocalize within the same vesicles. Although ODN enters cells that lack TLR9 (or express signal-defective TLR9 mutants), this uptake is sequence independent and does not influence vesicle formation. Together, these observations suggest that vesicular uptake of CpG ODN is associated with cell signaling. Third, the recognition of CpG DNA by hTLR9 is exquisitely sequence specific. Eliminating the CpG dinucleotide by inversion or methylation abrogates responsiveness. Moreover, the CpG flanking region determines whether an ODN will activate human cells, and concomitantly, whether it will trigger through hTLR9. These findings suggest that species-specific differences in the recognition of bacterial DNA evolved through diversification of TLR9.

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